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Ageing of human corneal and scleral collagen

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Thesis submitted for the degree of
Doctor of Philosophy
in the discipline of Biophysics

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To my mother and father
- my inspiration

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Abstract

It is commonly accepted that, on ageing, the body's proteins become structurally altered. One such protein predominating throughout the body is collagen. This long-lived protein forms a major part of the human cornea and sclera and it is within these tissues that the work of this thesis has revealed age-related structural and biochemical modifications of type I collagen. Using the highly sensitive technique of X-ray diffraction, the size of the intermolecular unit cell of the collagen has been demonstrated to increase in both tissues from birth to 90 years of age. Across the same age range, the volume of the fibril unit cell of corneal collagen has been found to decrease. In an attempt to account for the molecular changes, the concept of sugar-induced structural modification (glycation) was investigated using a colorimetric assay. This too was found to increase with tissue age as did the production of fluorescent cross-links (possibly intermolecular) on the collagen. These fluorescent structures were quantified by their fluorescence emission properties. Different sugars were also used, to compare their effects on the intermolecular unit cell volume. Since collagen is a family of proteins, the glycation of two different collagen types was studied. Several chemical compounds were also used to investigate the inhibition of in vitro glycation of collagen. The absence of an increase in intermolecular unit cell volume in the presence of these compounds suggests that it may be possible to control this particular consequence of ageing. The literature suggests that interfibrillar spacing is linked with corneal transparency. Proteoglycans are essentially involved in controlling this particular spacing and the work of this thesis has shown no significant loss of these macromolecules to the organ culture medium in which the tissue is housed before investigation. Tissue proteoglycan may be lost during the natural ageing of an individual.

Abbreviations

AG	Aminoguanidine
AGE	Advanced glycosylation end-product
AP	Amadori product
ASP	Aspirin
CS	Chondroitin sulphate
BDMA	N-Benzyl dimethylamine
DDSA	Dodecenyl succinic anhydride
DETAPAC	Diethylenetriaminepentaacetic acid
DS	Dermatan sulphate
EDTA	Ethylenediaminetetracetic acid
GAG	Glycosaminoglycan
HMF	Hydroxymethylfurfuraldehyde
HYPRO	Hydroxyproline
KS	Keratan sulphate
MNA	Methyl nadic anhydride
PEG	Polyethylene glycol
PAGE	Polyacrylamide gel electrophoresis
SB	Schiff base
SDS	Sodium dodecylsulphate
SFD	Specimen-to-film distance
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
M	molar
MgCl ₂	Magnesium chloride
mM	millimolar
ml	millilitre
mins	minutes
hrs	hours
M _r	relative molecular mass
r	correlation coefficient
μg	microgram
V/V	volume/volume
V	volt
W/V	weight/volume

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Chapter 1

Introduction

This thesis is concerned with the ageing of collagen within the human cornea and sclera and in particular how the collagen of these eye tissues is altered both structurally and biochemically with age. This chapter will deal with the structure and composition of both the cornea and sclera and will outline the aims of the experiments undertaken.

1.1 The human eye

Figure 1.1 illustrates a horizontal cross-section through a vertebrate eye. It is spherical in shape and in human adults has a diameter of approximately 24mm. The globe is covered posteriorly by the sclera which is the tough, white, opaque coat serving to protect the eye. Anteriorly the globe is covered by a transparent layer, the cornea. Light passes through the cornea and the transparent aqueous humor, which lines the posterior of the cornea, and eventually reaches another transparent structure, the lens. The lens serves to focus incoming images onto the retina. Posterior to the lens is another transparent medium, the vitreous which occupies approximately 90% of the total volume of the eye and not only provides strong structural support but also protects against mechanical impact.

1.2 Structure of the cornea

The cornea is composed of 5 distinct anatomical layers as illustrated in Figure 1.2. These layers lie parallel to the corneal surface and are further described below.

1.2.1 Epithelium

This is the outermost layer which contributes 8-10% of the total corneal thickness in mammals. Although the number of cell layers can vary from species to species the cell types are essentially columnar and squamous. The epithelium's primary function is, with the tear film, to provide a very smooth refracting surface at the front of the eye. Its barrier function reduces evaporation and minimises absorption of fluid from the tears, thus helping to maintain proper corneal hydration.

1.2.2 Basal lamina

Also known as Bowman's layer, this is a basement membrane underlying the epithelium. It consists of a disordered array of collagen fibrils and is a relatively thin membrane when compared to the total corneal thickness.

1.2.3 Stroma

The stroma comprises 90% of the total corneal thickness and consists of lamellae which are arranged such that they lie parallel to the surface [Jakus, 1954]. Within each lamella the collagen fibrils are of uniform diameter and lie parallel to each other but not to those of neighbouring lamellae. The normal human corneal stroma contains 78% water [Maurice, 1984] and the major structural constituents are collagen and proteoglycans, which comprise 12-15% and 1-3%, respectively, of the wet weight of the tissue. Non-collagenous structural proteins, soluble proteins, glycoproteins

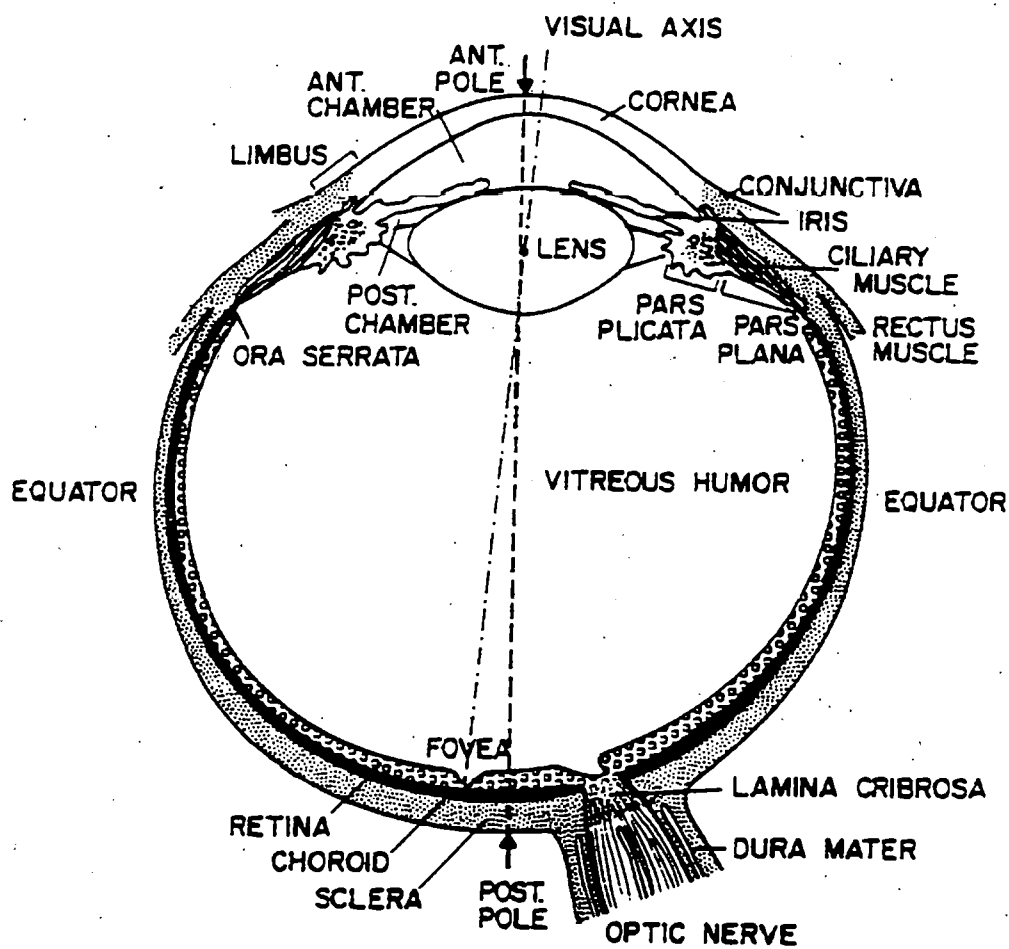


Figure 1.1 Diagrammatic representation of horizontal cross-section through human eye

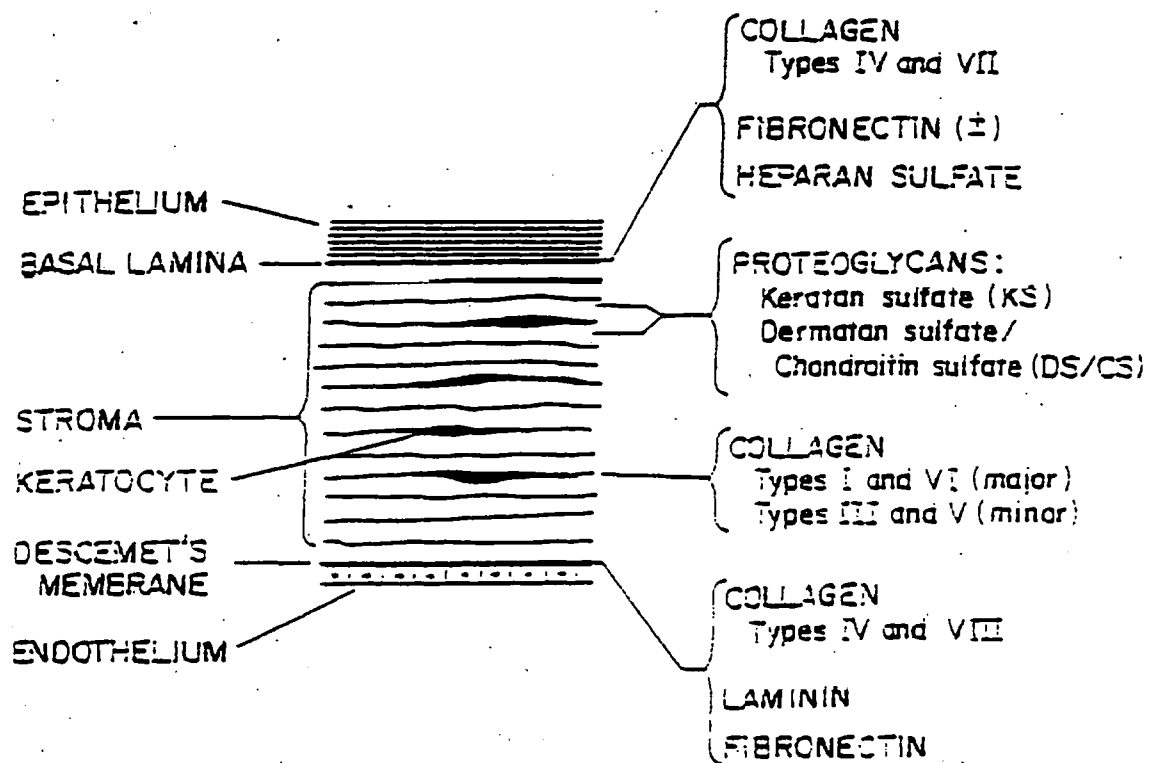


Figure 1.2 Meridional cross-section through the human cornea (from Biochemistry of the Eye, 1991)

and lipids are also present together with electrolytes, commonly sodium and chloride ions. These ions play an important role in corneal hydration [Maurice, 1984].

1.2.4 Descemet's membrane

This is a basement membrane produced by the endothelial cells [Kapoor et al., 1986]. The thickness varies with age from approximately $3\mu\text{m}$ in young humans to $9\text{-}10\mu\text{m}$ in adults [Murphy et al., 1984]. The membrane contains both laminin and fibronectin. The principal collagen type found here is type VIII [Labermeier and Kenney, 1983] although type IV has also been identified [Labermeier et al., 1983].

1.2.5 Endothelium

This is a single layer of hexagonally-shaped cells located on Descemet's membrane and found to be lining the posterior surface of the cornea. The corneal endothelium plays an important role in maintaining corneal dehydration and transparency by continuously pumping fluid and ions out of the stroma into the aqueous.

1.3 Structure of the sclera

Figure 1.3 illustrates the different architecture of the cornea and sclera. The sclera is less hydrated than the cornea so 75% of its dry weight is made up of collagen, 10% by other proteins and 1% by proteoglycans. Type I collagen predominates although types III and V exist in sclera. A greater proportion of type III collagen is found here compared with the cornea. There is a suggestion that type III imparts elasticity to the tissue. Type VI collagen has been found localised to the filamentous strands in the scleral interfibrillar matrix [Marshall and Lee, 1993]. As with the cornea, fibrous

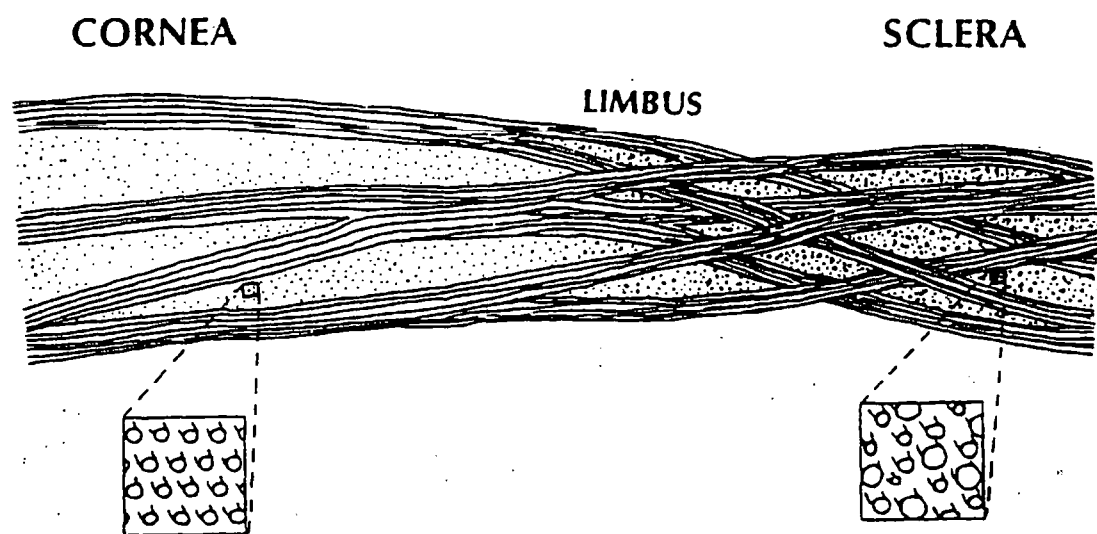


Figure 1.3 The structure of the cornea and sclera; the lamellae become increasingly interwoven towards the limbal and scleral regions (reproduced from *The Eye*, 1969)

bands of collagen run across the limbus. However, unlike the highly-organized collagen of the cornea, the sclera contains collagen fibres that divide and recombine, frequently giving the appearance of an interlaced structure. These bands are a continuation of the corneal lamellae. The sclera contains numerous elastic fibres running parallel to the collagen [Virchow, 1910]. Within sclera the collagen fibres are also of unequal diameter and can vary from 30-300nm [Schwarz, 1953a,b]. Unlike the cornea, the fibrils are covered by a thinner coating of ground substance and less proteoglycan is found here too. The sclera itself is almost avascular, however it does possess a looser outer coat in which there is a well-developed network of vessels near the corneal margin. Throughout the scleral tissue, fixed cells similar to those of the cornea exist together with pigmented cells commonly found in the choroid.

1.4 Corneal transparency

Although the outer protective coat of the eye is formed by both the cornea and sclera, it is the former tissue which is transparent to light. The cornea transmits approximately 90% of light in the visible spectrum. The need for corneal transparency is obvious, however the mechanism by which this is achieved has long been debated. The lack of blood and lymph vessels, the absence of myelin sheaths around the corneal nerves, and proper hydration of the stroma are certainly necessary. Other connective tissues have similar mixtures of collagen fibrils and ground substance, for example sclera, yet the corneal stroma remains transparent.

During the early stages of development the cornea and sclera possess collagen fibrils of equal diameter [Smelser, 1960] and both tissues appear to be translucent. As development progresses, the fibrils of the cornea become narrow and attain a uniform diameter of approximately 35-39nm [Craig et al., 1987] and the tissue remains transparent, unlike scleral tissue. The collagen fibrils of the sclera continue to grow and result in a large range of fibril diameters.

Maurice (1957) suggested that the tight packing and regular lattice arrangement of the corneal stromal collagen fibrils is responsible for corneal transparency. Light scattered by individual fibres is cancelled by destructive interference with scattered light from neighbouring fibres. As long as the fibres are regularly arranged and separated by less than a wavelength of light, the cornea will remain transparent. However, if the stroma becomes edematous, the space between the fibres increases, destructive interference no longer occurs, light is scattered and corneal transparency is reduced. It must be noted however, that a regular lattice arrangement of the collagen fibres does not occur in Bowman's layer. Goldman and Benedek (1968) concluded that significant light scattering did not occur unless there were appreciable regional fluctuations in refractive index. The corneal stroma does not scatter light because its collagen fibrils are small in diameter (approx 35-39nm), uniformly packed and closely spaced (approx 55nm).

1.5 Control of stromal hydration

The control of corneal stromal hydration is essential for transparency. Water accounts for 78% of the weight of the cornea, higher than most connective tissue elsewhere in the body. The hydration (H) can be described as the ratio of water (by weight) to dry weight of tissue. The physiological hydration of normal human cornea is taken to be 3.2 [Meek et al., 1991] and if this is increased, the thickness of the tissue increases too (at $H=6.8$ corneal thickness doubles). The mechanisms which play a role in the regulation of corneal hydration (illustrated in Figure 1.4) are :

- a. barrier function of the epithelium and endothelium - both these cell layers act as barriers to the movement of water and ions into the stroma.
- b. swelling pressure of the stroma - this is controlled by the glycosaminoglycans of the ground substance.
- c. ionic transport by the epithelium and endothelium - the mechanism in the endothelium is one of active transport of ions from the stroma into the

aqueous, with passive, secondary movements of water. This creates an osmotic gradient which balances the swelling pressure of the corneal stroma [Fischbarg et al., 1985].

d. intraocular pressure - when intraocular pressure exceeds the stromal swelling pressure epithelial edema occurs, the anterior stromal lamellae are thought to bear the stress of the intraocular pressure [McPhee et al., 1985].

e. evaporation of water from the corneal surface - evaporation of water from the tear film results in hypertonicity of the tears and draws water from the epithelial cells, and subsequently the stroma.

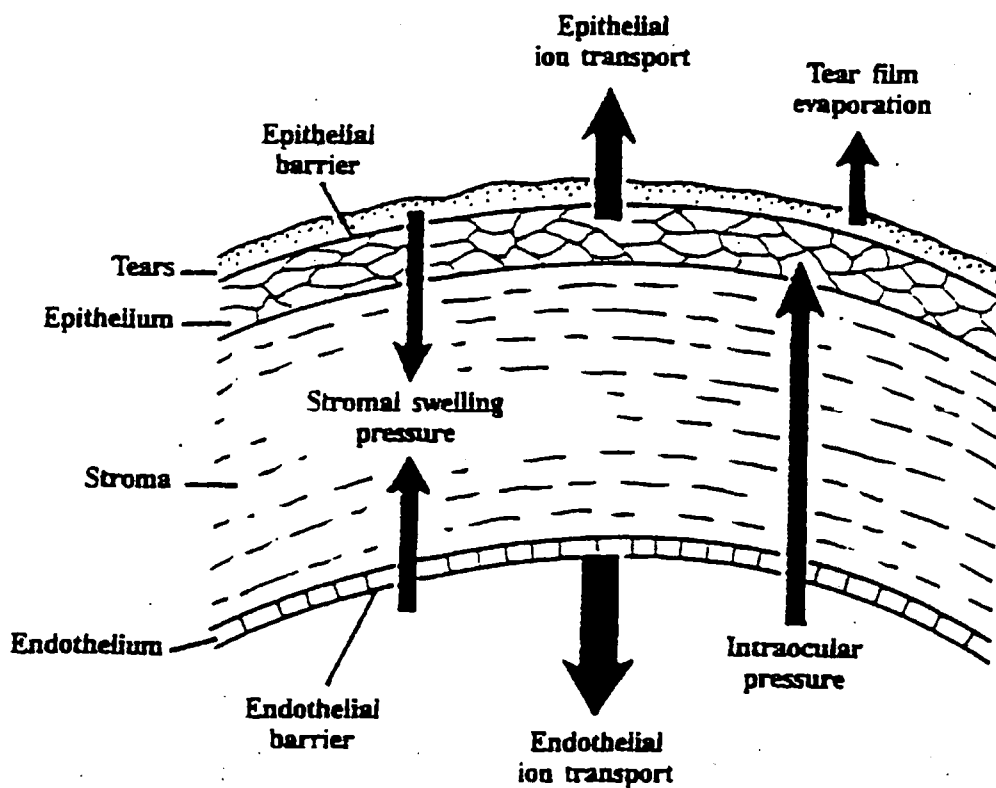


Figure 1.4 Diagrammatic representation of the control of corneal stromal hydration

1.6 Collagen

Collagen is a major constituent of all extracellular matrices and is of great importance structurally. The term collagen refers to a complex family of genetically distinct proteins. To date, seventeen types of collagen have been described and characterized to varying degrees.

1.6.1 The collagen molecule

The shape and most of the structural properties of a native collagen molecule are determined by its triple-helical domain(s). The fibril-forming collagens possess a single triple-helical domain which comprises more than 95% of the molecule (Figure 1.5).

Other collagens have multiple triple-helical domains and these may represent only a fraction of the mass of the molecule. A triple-helical or collagenous domain consists of three separate chains (α chains), each of which contains a characteristic sequence of amino acids twisted in the form of a left-handed helix [Ramachandran and Ramakrishnan, 1976]. The three helical chains are wrapped around one another to produce a higher-order tight, triple-helical structure of the molecule [Traub and Piez, 1971]. This conformation is stabilized by interchain hydrogen bonds. These are the bonds that are disrupted during thermal and chemical denaturation, resulting in unfolding of the molecule. Folding of the component α chains requires glycine as every third amino acid residue. The α chains exist therefore as a series of triplet Gly-X-Y sequences in which X or Y can be any amino acid. Frequently, X is proline and Y is hydroxyproline. A large number of hydroxyproline residues adds stability to the helical structure whilst lysine and its enzymatically-modified forms participate in covalent cross-linking between chains and molecules, and act as sites for sugar attachment.

The collagen molecules spontaneously self-assemble to form fibrillar structures in a characteristic quarter-stagger arrangement. When examined

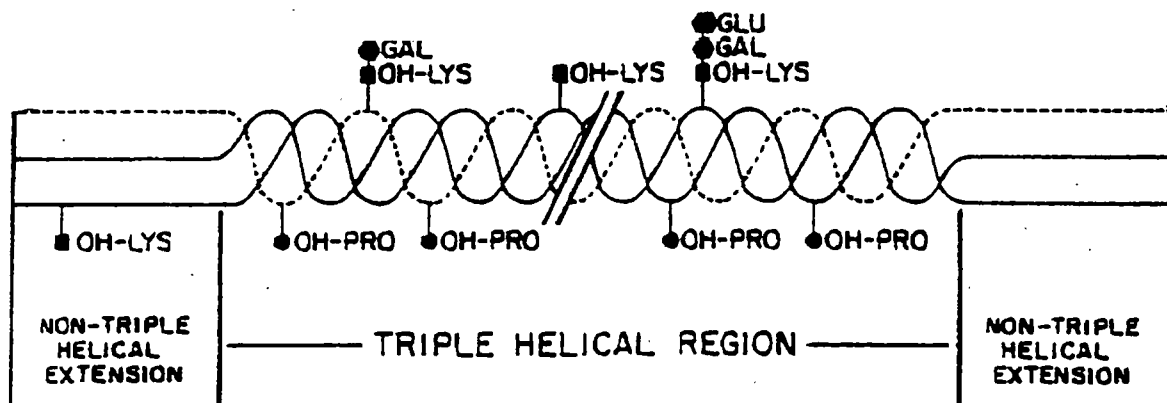


Figure 1.5 Diagram of the major characteristics of the type I collagen molecule. OH-PRO, hydroxyproline; OH-LYS, hydroxylysine; GAL, galactose; GLU, glucose (from Cell Biology of the Extracellular Matrix, 1991)

by electron microscopy, collagen fibrils exhibit a characteristic transverse banding pattern as shown in Figure 1.6.

The diagram illustrates the staggered packing arrangement of the molecules in the fibrils as suggested by Hodge and Petruska in 1963. Low angle X-ray diffraction patterns give a series of meridional reflections that indicate that the feature responsible for this banding pattern has a periodicity (D) of 67nm in rat tail tendon and sclera, whilst it has a value of 65nm in cornea. Since the individual collagen molecules are around 300nm in length, the periodicity has been attributed to an arrangement in which the molecules are axially staggered. Since the molecular length is not an exact multiple of $D=67\text{nm}$, there are abrupt changes in electron density at intervals of approximately $D/2$.

1.6.2 Posttranslational modifications

Proline incorporated into the Y position of the Gly-X-Y triplets of newly forming α chains can be enzymatically converted to 4-hydroxyproline. The enzyme involved in this reaction is prolyl hydroxylase which acts in the presence of molecular oxygen and the cofactors ascorbic acid and ferrous iron.

Lysine incorporated into the collagen chains can undergo several enzymatic conversions. Lysine residues in the Y position of Gly-X-Y triplets can be converted to hydroxylysine by the enzyme lysyl hydroxylase in the presence of molecular oxygen and the cofactors ascorbic acid and ferrous iron.

Hydroxylysine can undergo further posttranslational enzymatic modifications including glycosylation which results in the glycosylated derivatives galactosylhydroxylysine and glucosylgalactosylhydroxylysine. The best understood modification of lysine and hydroxylysine is the conversion to their respective aldehyde forms, allysine and hydroxyallysine. These chemically active compounds are necessary for the process of intramolecular and intermolecular cross-link formation [Tanzer, 1973] (see further in section 1.9).

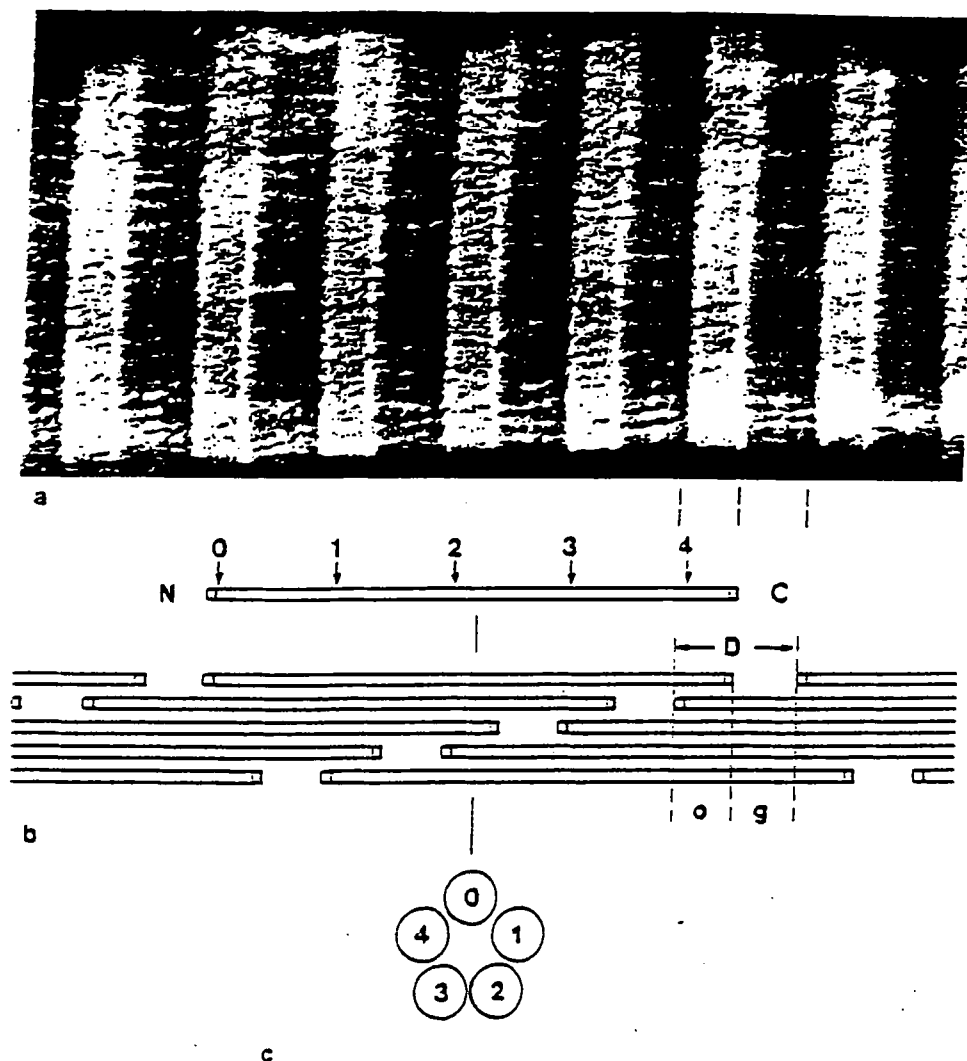


Figure 1.6 Staggered packing arrangement of molecules in the fibril suggested by Hodge and Petruska (1963). (a) Electron micrographic representation of a negatively-stained collagen fibril (b) Top - diagrammatic representation of a single collagen molecule, Bottom - illustration of the axial stagger between molecules that produces the D-period seen in (a). 'O' - overlap region, 'g' - gap region (c) Cross-section at overlap region showing the 5 molecules of the 5-stranded helical model

1.7 Collagen types

Type I collagen is the major fibril-forming collagen of the corneal stroma; it forms 52% of the corneal dry weight and consists of three polypeptide chains (each of M_r 100,000 kDa) arranged in a triple helix. Two different polypeptides are found to form type I collagen, $\alpha 1(I)$ and $\alpha 2(I)$. A molecule of type I collagen typically consists of one $\alpha 2(I)$ and two $\alpha 1(I)$ chains [Piez et al., 1963]. Type I collagen is also found in bone, tendon and skin.

Type III collagen is molecularly similar to type I collagen and is composed of three identical $\alpha 1(III)$ chains of M_r 100,000 kDa. It is found in the human corneal stroma (1% of the corneal dry weight) and has been shown to exist often in association with type I collagen molecules [Henkel and Glanville, 1982]. It is also present in skin, blood vessels and smooth muscle.

Type IV collagen has been found in the corneal basal lamina and Descemet's membrane [Pirie, 1951] and comprises about 1% of the corneal dry weight. It appears to be formed from two different kinds of polypeptide chain, $\alpha 1(IV)$ (M_r 180,000 kDa) and $\alpha 2(IV)$ (M_r 165,000 kDa).

Type V collagen is very similar to type I and forms about 8% of corneal collagen. It is often found in association with type I fibres in the corneal stroma. It is possible for types I and V to be present within the same fibre [Linsenmayer et al., 1986] and, depending on their distribution within the fibre, they may affect the tensile strength of the fibre.

Type VI collagen has been estimated to comprise approximately 30% of the total corneal collagen suggesting that it plays a very important role within the corneal stroma. Type VI collagen is a hybrid molecule of part triple helix and part globular protein, and rotary shadowing pictures of this collagen type show the molecules as dumbbell-shaped with two globular domains of approximately equal size linked by a long triple helical section. Although the function of type VI collagen is relatively unknown, it may serve as an attachment site for cells and may also act between collagenous and globular proteins thereby contributing to the organisation of stromal type I collagen fibrils and proteoglycans [Trueb and Bornstein, 1984].

1.8 Proteoglycans in the cornea

Proteoglycans make up the ground substance (matrix) between the cells and collagen of the cornea and sclera and are structurally very important. Essentially they consist of a core protein to which are covalently linked a number of repeating disaccharide units known as glycosaminoglycans (GAGS). These repeating units are polyanionic as a result of the presence of many carboxyl and sulphate groups. The disaccharide typically consists of a hexosamine (either D-glucosamine or D-galactosamine) and a uronic acid (either D-glucuronic acid or L-iduronic acid). GAGs are linked covalently to proteins to form proteoglycans and thereby create subunits that can form large proteoglycan aggregates. Under physiological conditions, the carboxyl and sulphate groups on the sugars are ionised, thereby producing highly negatively charged molecules. This feature contributes to the function of the proteoglycans in connective tissues since it produces highly hydrated, space-filling molecules.

In the cornea, chondroitin sulphate exists as a hybrid molecule of chondroitin-4-sulphate and a small proportion of dermatan sulphate, it is therefore referred to as DS/CS [Scott, 1988]. The bulk of GAG in the corneal stroma is keratan sulphate (KS, approximately 65%). The KS proteoglycan is often referred to as lumican [Kjellen and Lindahl, 1991] and can exist in two forms. Although both forms have only one keratan sulphate side chain, the length of this chain varies and serves to distinguish the two types. The remainder of GAG (approximately 30%) is DS/CS. There are two types of DS/CS proteoglycan - they contain one and two DS/CS GAG chains and are referred to as 'decorin' and 'biglycan' respectively [Kjellen and Lindahl, 1991]. Borchering et al and others found that collagen fibres within the cornea appear to be covered with a dense coat of granules i.e. small highly charged KS molecules. It has been suggested that this coat prevents association between individual fibres hence it is likely that KS plays an important role in the spacing of collagen fibres by mutual repulsion of the charged KS molecules.

Human sclera contains predominantly dermatan sulphate proteoglycan, both a large and small type. The latter is similar to that found in cornea. Chick sclera has been shown to possess the DS/CS proteoglycan known as decorin (M_r 43 000 kDa) and a second larger proteoglycan known as aggrecan ($M_r > 1 \times 10^6$ kDa).

Chondroitin sulphate proteoglycan (aggrecan) plays an important role, as in cartilage, in endowing the sclera with compressive stiffness and the ability to sustain loading resulting from the intraocular pressure. Decorin is localised at specific sites along collagen fibrils in vivo [Scott, 1988] and it is the core protein that is essential for binding to collagen. In cornea KSPG preferentially localises on the collagen surface near the 'a' and 'c' bands while DS/CSPG preferentially localises near the 'd' and 'e' bands [Scott, 1988] as illustrated in Figure 1.7.

Approximately 12% of the stromal proteoglycans are in an ordered arrangement associated with the specific binding sites shown in the diagram [Meek et al., 1986]. The remaining proteoglycans seem to be distributed diffusely throughout the interfibrillar space.

1.9 Collagen cross-linking

Covalent cross-linking based on aldehyde formation from lysine or hydroxylysine side chains, begins between adjacent molecules. The intermolecular cross-links stabilise molecular arrangement within collagen fibrils and, in this way, confer their stability and their physical and mechanical properties to connective tissues. These cross-links are essential to give fibrils the necessary tensile strength to perform their structural role.

1.9.1 Enzymically-derived cross-links

Lysyl oxidase is the only known enzyme involved in the cross-linking of collagen. It contains both a copper ion and an aromatic carbonyl as cofactors. The enzyme initiates the formation of cross-links in the

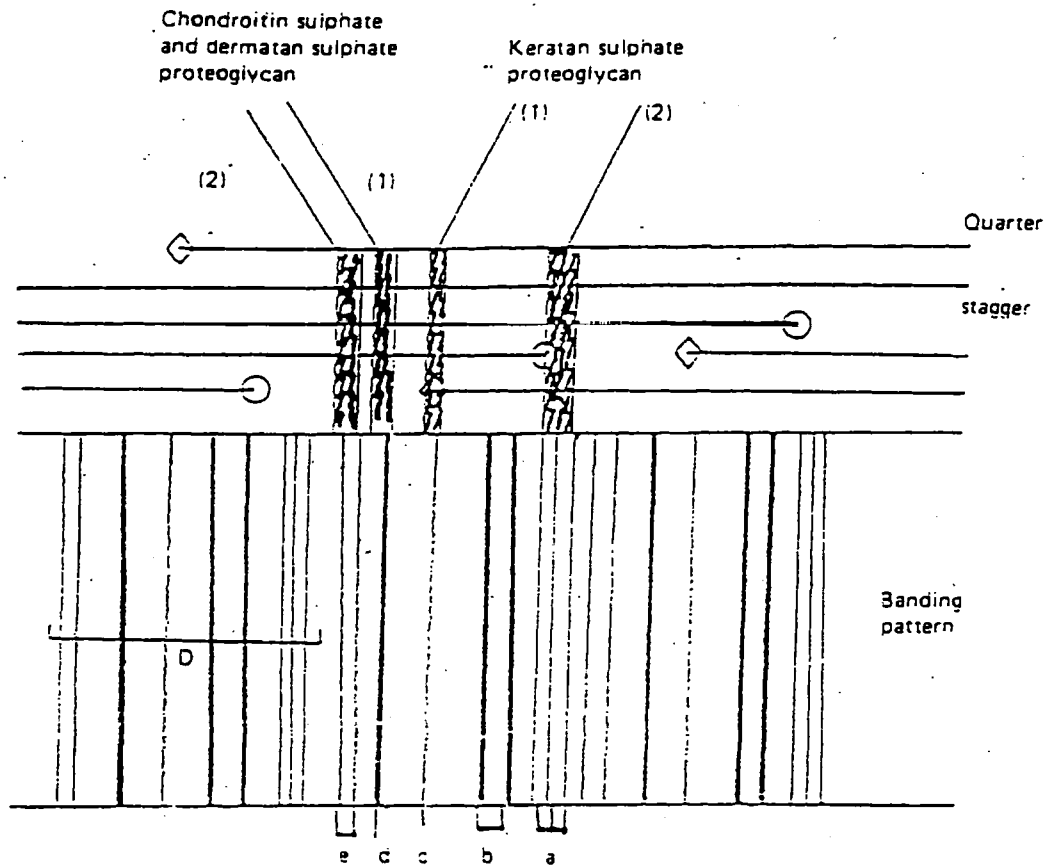
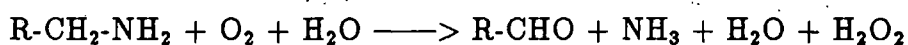


Figure 1.7 Diagrammatic representation of proteoglycan binding sites within the D period of type I collagen (from rabbit cornea). The a-e banding pattern is shown in the lower portion of the diagram against the arrangement of collagen molecules in quarter stagger. The locations of the proteoglycans in the upper part of the diagram correspond to the a,c,d and e banding patterns (from Biochemistry of the Eye, 1991)

extracellular space by catalysing the oxidative deamination of ϵ -amino groups of specific lysyl and hydroxylysyl residues to the corresponding aldehydes (allysine and hydroxyallysine respectively) according to the following reaction:



These side-chain aldehyde groups spontaneously condense with ϵ -amino groups of other lysyl or hydroxylysyl residues. In addition to the lysine-derived cross-links, another covalent cross-link arising from disulphide bonding exists in some collagens.

Within the group of intermolecular cross-links there is a subdivision, namely, cross-links laid down in newly formed collagen and those formed during maturation. The cross-links of young tissue are also known as 'reducible' cross-links as a result of their ability to be reduced by borohydride (e.g. hydroxylysinohydroxynorleucine, hydroxylysinonorleucine and lysinonorleucine) whereas those of mature tissue cannot be reduced and appear to be stable to high temperatures and low pH treatment. Hence they are referred to as 'mature' cross-links, e.g. pyridinoline (illustrated in Figure 1.8) which is also known to accumulate with age [Moriguchi and Fujimoto, 1978].

1.9.2 Glycation-derived cross-links

Collagen also contains nonenzymically glycosylated lysine and hydroxylysine residues [Eyre et al., 1984] resulting from the spontaneous reaction of a reducing sugar, commonly glucose, with any free amino group available on the protein. This reaction is also known as glycation. The sugar-induced cross-linking is thought to occur possibly between the triple helices i.e. inter-triple-helical cross-links [Bailey and Kent, 1989] as suggested by Figure 1.9.

Glycation forms the first step of a reaction first shown to occur in food, the Maillard reaction. This proceeds in three steps which can be subdivided

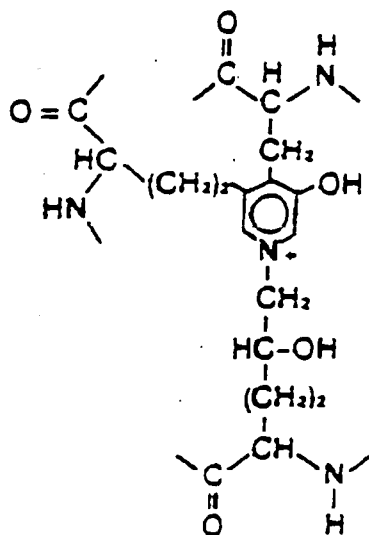


Figure 1.8 The chemical structure of the mature cross-link pyridinoline

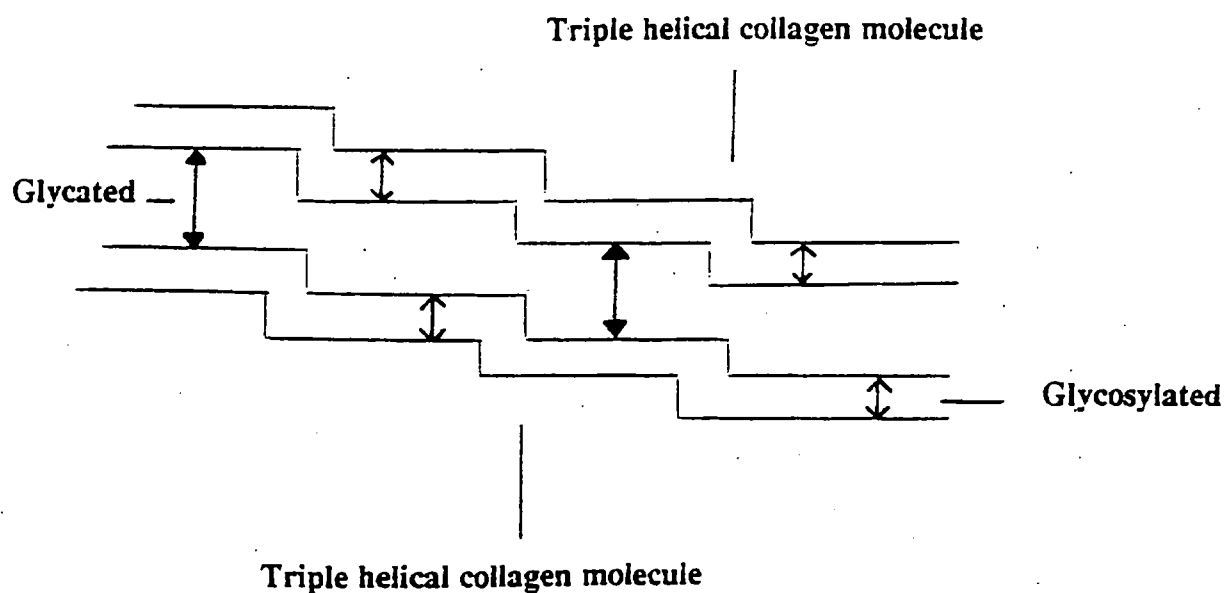


Figure 1.9 Cross-linking of collagen suggested by Bailey and Kent (1989)

into an initiation, propagation and termination phase (Figure 1.10).

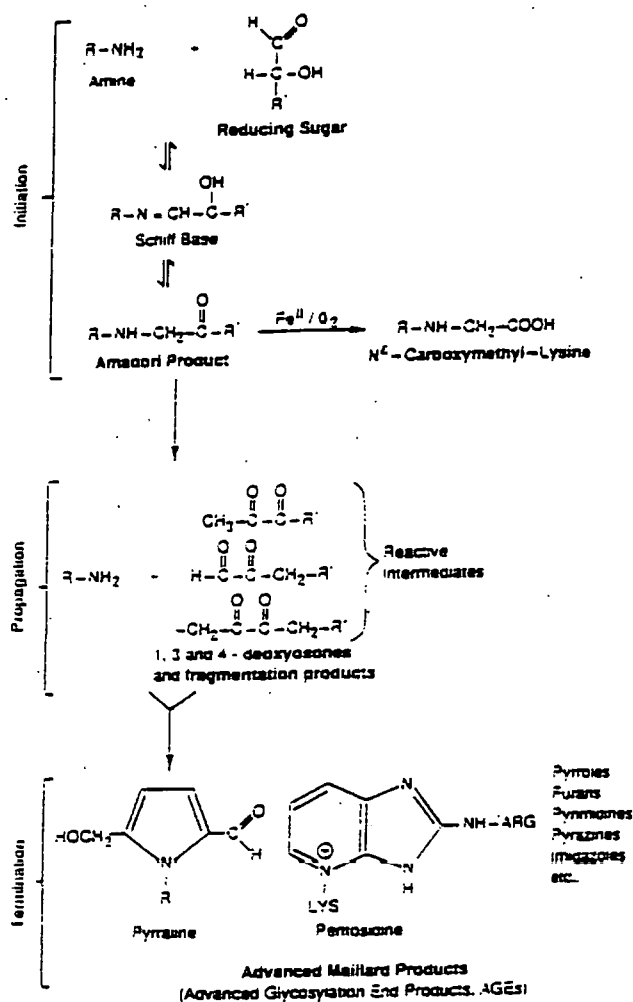


Figure 1.10 General scheme of the Maillard reaction - the initial step is referred to as glycation (or nonenzymic glycosylation)

Initiation - The reaction is initiated by nonenzymic condensation of a reducing sugar with an amine resulting in the formation of an Amadori product (AP). This is the product that many chemical assays for glycation measure to determine 'amount' of protein glycation. The initiation step itself is generally referred to as glycation however, terms such as glucation, fructation and ribation are also used to indicate the attachment of specific sugars like glucose, fructose and ribose respectively. The Amadori product can then either undergo oxidative fragmentation to irreversibly produce

carboxymethyllysine (CML) or it may dissociate to regenerate the intact amine ($R-NH_2$) while the sugar undergoes dehydration to form 1-, 3- or 4-deoxyones. Only 3-deoxyglucosone has actually been found in vivo and the literature suggests that a protein-bound carbonyl is also produced as a post-Amadori product [Liggins and Furth, 1993].

Propagation - The dicarbonyl compounds formed are highly reactive and can propagate the Maillard reaction and induce irreversible molecular damage by forming heterocyclic protein adducts which have been suggested as both intra- and intermolecular cross-links [Kato et al., 1989].

Termination - The protein adducts that are formed are thermodynamically-stable and can thereby terminate the Maillard reaction. Generally, they are referred to as advanced glycation end-products (AGEs) and their structures in proteins are by and large unknown.

The rate of glycation and in turn the level of protein modification, depends on:

1. the sugar involved
2. the concentration of the sugar and the percentage in the open-chain form
3. the duration of exposure to sugar
4. the temperature and nature of the incubation medium
5. the half-life of the protein involved

1.9.3 Glycating agents

Although glucose is the most abundant sugar in the living tissue, it has a low reactivity. Sugar phosphates (e.g. glucose-6-phosphate, fructose-6-phosphate) and carbohydrates with short carbon chains (e.g. ribose) have a higher reactivity.

In vitro studies show that fructose is a more potent glycating agent than glucose [Bunn and Higgins, 1981] and is as much as ten-fold more efficient at forming AGEs [Raza and Harding, 1991]. Although there is no simple assay for quantifying AGEs, the potential dangers of hyperglycaemia can be

assessed by analyzing for the glucose AP (which is commonly a ketone). The fructose AP of any protein is an aldehyde (Figure 1.11) and it is likely that an assay such as the thiobarbituric acid assay may underestimate the extent of fructation and ribation, i.e. reaction of a protein with fructose and ribose respectively. These sugars can however be used to accelerate the rate of glycation when performing in vitro studies in order to speed up the production of AGEs under in vitro conditions.

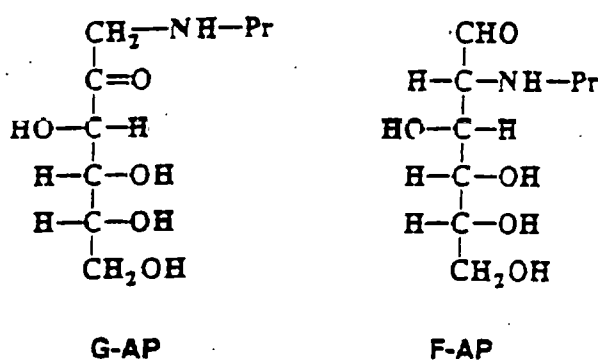


Figure 1.11 The structures of the glucose and fructose Amadori products (G-AP and F-AP, respectively). Pr represents the rest of the protein molecule

Since a greater proportion of ribose exists in the open chain form in solution when compared to glucose and fructose, it can react more readily with a protein containing sites available for this reaction. When compared to glucose, fructose contains a greater quantity of the open chain form in solution. In addition, glucose has a stable ring structure as a result of the equatorial orientation of its hydroxyl groups. This stable ring structure greatly retards the rate at which glucose reacts with protein molecules. The significance of such stability means that high concentrations of glucose, a major metabolic fuel, and proteins can co-exist in various tissues.

1.9.4 Fate of glycosylated proteins

Following glycation, a long-lived protein such as collagen may take several routes as illustrated in Figure 1.12. It can be converted to the Schiff base which can dissociate to give free protein; it may be catabolised to produce amino acids; it can be oxidised to produce reactive compounds such as 3-deoxyglucosone; or the protein can undergo further reactions which include oxidative reactions forming AGEs.

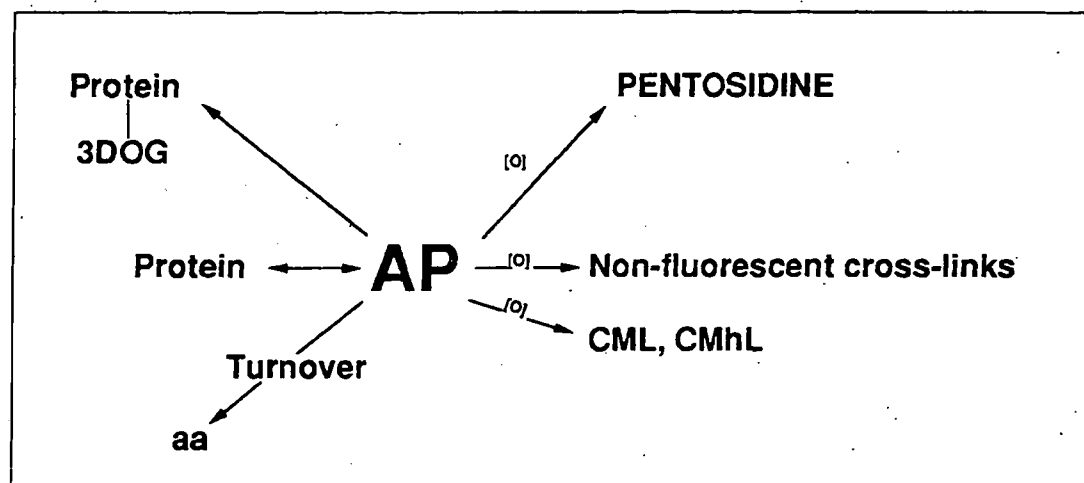


Figure 1.12 The various pathways that a glycosylated protein can take. 3-DOG, 3-deoxyglucosone; aa, amino acid; CML, carboxymethyllysine; CMhL, carboxymethylhydroxylysine; [O], oxidative reaction

1.10 Advanced glycation end-products (AGEs)

The AP (Amadori product) resulting from the glycation of a protein may react with other APs or free amino groups on this protein or a different protein. The result is the slow formation of AGEs. Since collagen is a long-lived protein these products are able to accumulate in vivo such that they can induce irreversible damage to the protein and can also be detected as well as quantified. Such structures are not yet fully characterised and little is known of their properties. Although the glycation-derived cross-link pentosidine has fluorescent properties, the majority of glycation-derived products are likely to be non-fluorescent.

AGEs have been difficult to characterise since they appear to be

- 1) relatively complex
- 2) susceptible to destruction during acid hydrolysis
- 3) produced in low yields
- 4) difficult to isolate since artefacts may be generated during isolation procedures.

Three types of AGE have been identified one of which is discussed below since, from the studies carried out in this thesis, a similar structure may well exist in human cornea and sclera.

1.10.1 Pentosidine

This fluorescent cross-link, was first isolated from human dura mater collagen in 1989 and has been characterised by Sell and Monnier. The concentration of the cross-link was found to increase with tissue age and was present in noticeably higher concentrations within diabetic tissues [Sell and Monnier, 1990]. Trace amounts of pentosidine have also been found in human skin collagen and lens protein where its concentrations have also increased with age [Dyer et al., 1991].

Pentosidine, illustrated in Figure 1.13, is a cross-linking structure resulting

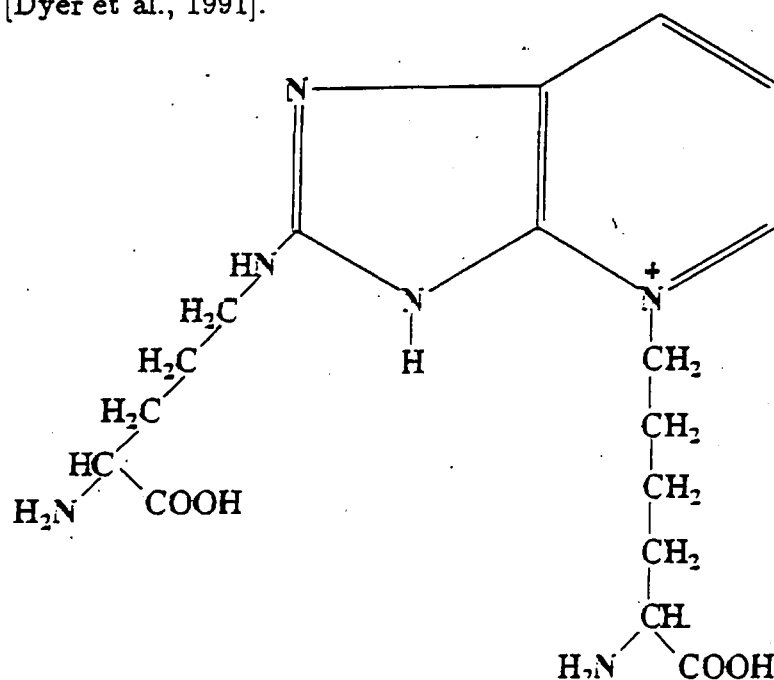
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Figure 1.13 The structure of the proposed cross-linking structure pentosidine

1.11 Physiological consequences of glycation on the ageing of collagen

The formation of the SB and the AP can result in an alteration of the isoelectric point of the protein and any changes in charge will lead to conformational changes of the collagen molecule. Though some investigators [Trueb, 1984] doubt if the low levels of glycation products found in vivo could result in significant physical changes in collagens, others point out that glycation could take place at biologically important sites on the molecule, for example the collagenase site or glycoprotein-interacting

sites [Bailey et al., 1989].

Glomerular basement membrane contains type IV collagen and in vitro glycation of this has been shown to affect the flexibility of the membrane and the permeability of the capillaries [Garlick et al., 1988]. Binding of fibronectin and heparan sulphate to basement membrane collagens is crucial in filtration and it has been reported that there is a reduction in the affinity of proteins to in vitro glycated type IV basement membrane collagen [Tarsio et al., 1987]. Glycation of the main collagenous domain NC1 (located at the carboxyl end of type IV collagen molecules) may well interfere with normal assembly of type IV collagen in diabetes mellitus and is possibly related to abnormal basement membrane function in this condition.

In vitro glycation of collagen has been shown by many workers (Yue et al., 1983; Brownlee et al., 1986; Tanaka et al., 1988) to alter its stability, solubility and digestibility by enzymes. Although earlier work reported an increase in the in vivo glycation of skin, basement membrane and tendon collagen with age, recent studies reveal only slight increases with tissue age [Patrick et al., 1990]. It has been suggested however, that the discrepancies between the results may well be due to differences in the methods used to assay for glycation.

The alterations in collagen cross-linking, rigidity, browning, fluorescence and a reduced susceptibility to proteolysis have been used as markers for AGEs and have been shown to increase in diabetes [Monnier et al., 1986]. It has yet to be determined whether such changes are due solely to glycation-induced AGEs or whether free radicals play a role [Jones et al., 1986].

1.12 Inhibition of glycation and AGE formation

The reactions that result in the formation of AGEs have been found to require oxygen and trace metal ions [Fu et al., 1992]. Metal ion chelators

can therefore inhibit these reactions as can antioxidants. Although, within the body, there may exist natural mechanisms to protect against such protein damage, at present there is considerable interest in pharmacological compounds (particularly aspirin) which may reduce or prevent glycation and AGE formation *in vivo*.

1.13 X-ray diffraction

The technique of X-ray diffraction can be employed to investigate regularly-arranged tissue structures such as collagen molecules and collagen fibrils within the cornea. It is the regularity of such structures that allows a characteristic X-ray diffraction pattern to be obtained. Using this highly sensitive technique it is possible to calculate the mean collagen interfibrillar spacing and also the mean intermolecular spacing.

The great advantage of this specialised technique is that the corneal and scleral tissues can be examined as close to their natural states as possible since there is no requirement for processing as in electron microscopy. The only tissue preparation involved is that of setting the corneal and scleral hydrations as close as possible to their physiological values prior to exposure to the X-ray beam. Again, in contrast to electron microscopy, the pattern arising from exposure of the specimen to the X-ray beam is automatically averaged for the dimensions of that beam. Electron micrographs of tissue sections exhibit areas of varied structure hence the interpretation can be more difficult than with X-ray patterns.

1.13.1 Equatorial and meridional diffraction patterns

A typical X-ray diffraction pattern from human cornea using a wide-angle camera is shown in Figure 1.14 along with the pattern obtained from a human cornea using a low-angle camera.

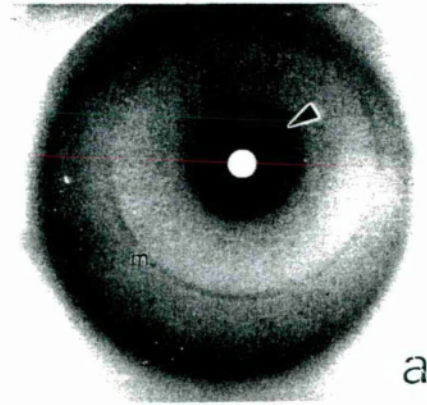


Figure 1.14 Typical X-ray diffraction patterns from the human cornea. (a) The high angle pattern from human cornea. The first order reflection derived from the intermolecular spacing is indicated by an arrow and corresponds to a spacing of approximately 1.80 nm, the outer ring (m) derives from the mylar window of the X-ray cell. (b) The low angle pattern from human cornea. The first order equatorial reflection, indicated by an arrow, is derived from the approximately 57 nm interfibrillar spacing

Figure 1.14(a) shows that the high angle equatorial diffraction pattern of cornea is a diffuse ring (low exposure). This arises from the lateral packing of the collagen molecules within the fibrils. Figure 1.14(b) shows that the low angle equatorial diffraction pattern (again at low exposure) consists of a single reflection. The pattern arises from regularity in the side-to-side packing of the uniform collagen fibrils [Goodfellow, 1975]. In bovine cornea, the pattern is circular which implies that the collagen fibrils are orientated in approximately equal numbers in all directions in the plane of the cornea. On further inspection of the pattern it is found to contain four lobes, these suggest that the fibrils are preferentially orientated in a superior-inferior manner. The width of the ring is related to the range of interfibrillar spacings present and hence the mode of packing of the fibrils within the cornea.

It is not possible to obtain X-ray reflections from the interfibrillar spacing in sclera since the collagen fibrils within this tissue vary greatly in size.

1.14 Aims and objectives of this thesis

Changes in the structural organisation of the collagen within the cornea and sclera can affect both the physical and optical properties of these tissues. In an attempt to investigate the causes of such changes, age-related studies were performed on both human corneas and scleras and also on the incubation medium in which they are housed. The aims of this work have been to investigate :

- 1) using SDS-PAGE and electron microscopy, the possible leaching (age-related or time-related) of proteins/proteoglycans from the corneoscleral discs to the incubation medium housing them
- 2) using X ray diffraction, the age-related changes in both the fibrillar and molecular organisation of type I collagen in the human cornea and sclera
- 3) using biochemical assay, the occurrence of non-enzymic glycosylation (glycation) within corneal and scleral collagen and the changes with tissue age

- 4) using biochemical assay, the occurrence of AGEs in both tissues and any alterations in their levels with tissue age
- 5) using a combination of biochemical techniques and X-ray diffraction, the ability to reproduce the results obtained by using in vitro set-ups (i.e. inducing glycation and then observing the effect on the molecular parameters)
- 6) the susceptibility of different collagen types to sugar-induced modifications
- 7) the effects of various inhibiting compounds on the level of glycation and the formation of AGEs in vitro

Chapter 2

Materials and Methods

The chemicals and materials used in the investigations are listed below together with their suppliers.

X-ray diffraction

X-ray film	Caeverken, Strangnass, Sweden
PEG (mwt 20,000)	BDH Laboratory Supplies, Poole, U.K.
14 kDa cut-off dialysis tubing	Medicell International Ltd, London, U.K.

Hydroxyproline assay

Hydrolysis tubes	Sarstedt Suppliers, Leicester, U.K.
Hydroxyproline	Sigma Chemical Co. Ltd, Dorset, U.K.
Perchloric acid	Sigma Chemical Co. Ltd, Dorset, U.K.
Chloramine T	BDH Laboratory Supplies, Poole, U.K.
2-Methoxyethanol	Aldrich Chemical Co. Ltd, Dorset, U.K.
Citric acid monohydrate	BDH Ltd, Poole, U.K.
Glacial acetic acid	BDH Ltd, Poole, U.K.
Sodium acetate trihydrate	BDH Ltd, Poole, U.K.
p-Dimethylaminobenzaldehyde	Aldrich Chemical Co. Ltd, Dorset, U.K.

Thiobarbituric acid (TBA) assay

Oxalic acid	BDH Ltd, Poole, U.K.
2-TBA	Sigma Chemical Co. Ltd, Dorset, U.K.
TCA	BDH Ltd, Poole, U.K.

Polyacrylamide gel electrophoresis

Acrylamide	BDH Ltd, Poole, U.K.
N,N',methylenebisacrylamide	BDH Ltd, Poole, U.K.
TEMED	BDH Ltd, Poole, U.K.
Ammonium persulphate	BDH Ltd, Poole, U.K.
β -mercaptoethanol	Sigma Chemical Co. Ltd, Dorset, U.K.
SDS	BDH Ltd, Poole, U.K.
Tris buffer	Sigma Chemical Co. Ltd, Dorset, U.K.
Molecular weight markers	Sigma Chemical Co. Ltd, Dorset, U.K.
Coomassie blue	Sigma Chemical Co. Ltd, Dorset, U.K.
Alcian blue 8GX	Sigma Chemical Co. Ltd, Dorset, U.K.
Photographic film (Ilford Pan F ISO 50)	Ilford Ltd, Mobberley, Cheshire, U.K.

Glycosaminoglycan assay

Alcian blue	Sigma Chemical Co. Ltd, Dorset, U.K.
Chondroitin sulphate	Sigma Chemical Co. Ltd, Dorset, U.K.

Electron microscopy

Agar 100 resin	Agar Scientific Ltd, Essex, U.K.
DDSA	Agar Scientific Ltd, Essex, U.K.
MNA	Agar Scientific Ltd, Essex, U.K.
BDMA	Agar Scientific Ltd, Essex, U.K.
Cuprolinic blue stain	BDH Ltd, Poole, U.K.

In vitro glycation

Collagen type I from calf skin

Collagen type I from bovine achilles tendon

Collagen type IV from human placenta

Collagen type I from rat tail

All collagen types supplied by Sigma Chemical Co. Ltd, Dorset, U.K.

Enzymes

Papain (Papainase; EC 3.4.22.2) Sigma Chemical Co. Ltd, Dorset, U.K.

Collagenase (EC 3.4.24.8) Sigma Chemical Co. Ltd, Dorset, U.K.

Other chemicals

Aminoguanidine Sigma Chemical Co. Ltd, Dorset, U.K.

Acetylsalicylic acid Sigma Chemical Co Ltd, Dorset, U.K.

Diethylenetriaminepentaacetic acid Sigma Chemical Co Ltd, Dorset, U.K.

2.1 Corneal supply and storage

The cultured human corneas and scleras were obtained from the Corneal Transplant Service Eye Bank, U.K. They were received as corneoscleral discs and stored at 34°C in an incubation medium comprised essentially of Eagles MEM with Earle's salts and HEPES buffer, 2% foetal calf serum, 2mM L-glutamine, 24mM sodium bicarbonate, 100 units ml⁻¹ penicillin, 0.1mg ml⁻¹ streptomycin and 0.25µg ml⁻¹ amphotericin B [Armitage and Moss, 1990]. These corneas were assumed to represent 'normal' individuals, unless the clinical details indicated otherwise, and had been rejected by the Eye Bank as unsuitable for grafting because of low endothelial cell counts and/or large arcii. The scleral rim was separated from the corneal disc using a metallic cork borer. The epithelial and endothelial cell layers were then removed by gently scraping with a scalpel blade. The tissues were tightly wrapped in cling film to minimise changes in hydration and stored at -40°C until used for investigation. The incubation media were also stored frozen for future analyses.

2.2 X-ray diffraction

Both high and low angle X-ray diffraction patterns for human corneas and scleras were recorded at the SERC high intensity synchrotron source at Daresbury, U.K. Since structural parameters such as interfibrillar and intermolecular spacings are strongly dependent on tissue hydration, before X-ray measurements were made, the corneal and scleral hydrations were set by placing the corneal discs into 14 kDa cut-off dialysis tubing and immersing them in a solution of 1.5% PEG containing 0.15M sodium chloride (for human corneas) or 2% PEG containing 0.15M sodium chloride (for human sclera). Equilibration was carried out for 4 days at 4°C.

The tissues were then brought to room temperature, removed from the dialysis tubing, weighed and placed in air-tight cells. The specimens were examined by passing the X-ray beam through the central region of the corneal button. Data were obtained on the interfibrillar spacing and the intermolecular spacing of the same specimens (however, for scleral tissue only an intermolecular measurement was possible).

High angle diffraction

High angle diffraction was carried out at station 7.2b. The wavelength of the X-rays was 0.1488nm and the beam was collimated to a diameter of 0.5mm. Exposure times of 3-4 minutes were used and the specimen-to-film distance was approximately 12cm. The camera was calibrated using the 0.305nm lattice reflection of calcite. Measurement of the first order diffraction ring from the packing of the collagen molecules allows the first order Bragg spacing to be obtained. The Bragg spacings were increased by a factor of 1.11 to obtain the mean centre-to-centre intermolecular spacing in the cornea; this factor relates to the 'pseudo-hexagonal' packing reportedly present in collagen fibrils [Maroudas et al., 1991].

Low angle diffraction

Low angle diffraction was carried out at station 8.2. The wavelength of the X-rays was 0.154nm and the beam dimensions were 4mm x 0.5mm (focussed in the vertical direction). Exposure times of 2 minutes were used and the specimen-to-film distance was approximately 3m. The camera was calibrated using the 67nm axial periodicity of rat tail tendon. Measurement of the first order diffraction ring from the packing of the collagen fibrils within the corneal stroma allows the first order Bragg spacing to be obtained. The Bragg spacing is increased by a factor of 1.12 to give the mean centre-to-centre spacing of the collagen fibrils. This factor arises from the most probable packing arrangement present in the corneal stroma [Worthington and Inouye, 1985].

After exposure the tissues were re-weighed to ensure that their hydration had remained fairly constant. Diffraction patterns were recorded on Caeverken AB photographic film.

The tissue hydration (H) was calculated following X-ray diffraction using the formula :

$$H = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}}$$

2.2.1 X-ray pattern analysis

The X-ray patterns obtained for each tissue were digitised using an Ultrascan XL Laser Microdensitometer (LKB Instruments, Gaithersburg, MD) which produced linear scans across the diffraction rings. Measurements were made after taking account of local background scatter. A typical densitometer scan of the X-ray diffraction pattern from a middle-aged human cornea in a wide angle camera is illustrated in Figure 2.1.

Interpretation of the X-ray patterns involves use of Bragg's equation :

$$n\lambda = 2d\sin\theta$$

where n is the order of the reflection, λ is the wavelength of the radiation, θ is half the angle of scatter, d is the Bragg spacing, and n is the order of the reflection. As mentioned earlier, the specimen-to-film distance (SFD) can be accurately obtained by using a calibration specimen of known spacing - calcite ($d=0.305\text{nm}$) for the high angle studies and rat tail tendon ($d=67\text{nm}$) for the low angle ones. For the calibration specimen λ and d are known, therefore by using Bragg's equation (for $n=1$) it is possible to obtain θ . The SFD can be calculated by measuring the radius of the X-ray pattern. Once the SFD is known, it is possible to calculate θ by measuring the diameter of X-ray patterns from human specimens, this allows d the Bragg spacing to be obtained for all specimens.

LKB UltraScan XL

Run by Operator: # 1 Run ID # = 1

Track = 1 of 1 X-position = 126.6 X-width = 2
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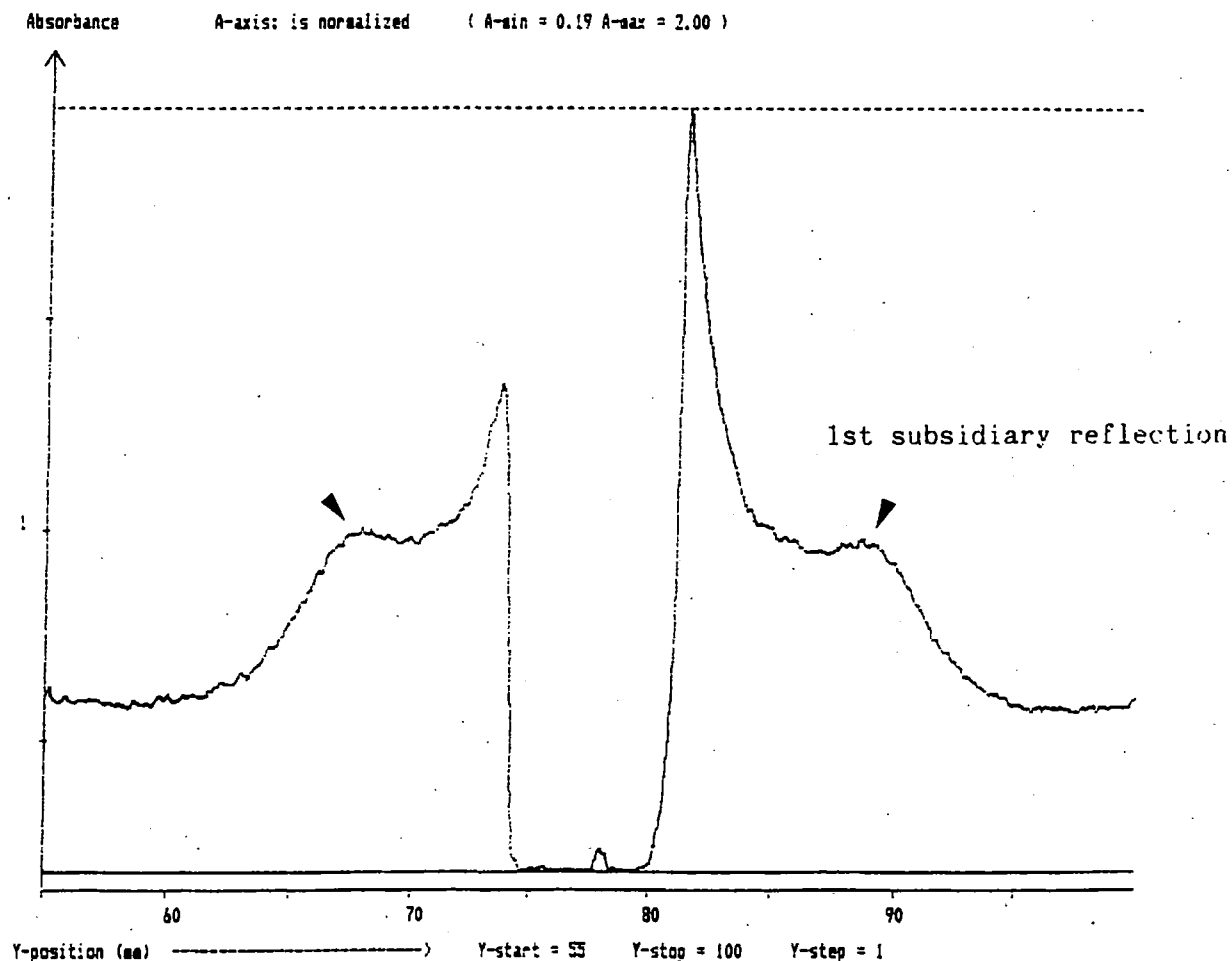


Figure 2.1 Densitometer scan produced from analysis of an X-ray diffraction pattern of a middle-aged human cornea

2.3 Extraction of corneal and scleral collagen

Several extraction steps were required to isolate collagen from the cultured corneas and scleras prior to biochemical measurement of glycation and fluorescence. The procedure below would be expected to isolate predominantly type I collagen.

Extraction in 0.15M sodium chloride for 3 days at 4°C removed soluble material from the tissue and this was followed by extraction in a 4M guanidine hydrochloride/0.15M sodium chloride mixture for 3 days which removed more strongly bound materials such as proteoglycans [Wall, 1990]. Several washes in 0.15M sodium chloride were required to remove any traces of guanidine hydrochloride. Throughout the entire washing procedure the tissues remained at 4°C and the solutions were constantly stirred. Protease inhibitors - 2.5mM benzamidine hydrochloride, 0.01% trypsin inhibitor and 5mM disodium EDTA - were added to all the extraction solutions. The collagen from the tissues was solubilised by digesting with papain (0.5mg ml⁻¹ citrate buffer pH 6) at 45-50°C for 24 hrs. Once digested, the samples were centrifuged at 9000rpm for 10 mins and the supernatant was stored frozen for biochemical analysis.

2.3.1 Digestion of collagen

Dialysed corneal and scleral tissue samples were subjected to several extractions (described in section 2.3) in order to remove proteoglycans such that type I collagen predominantly remained. The collagen obtained was then digested with papain (0.5mg/ml citrate buffer, pH 6). The various different collagen types obtained from the manufacturer were digested with collagenase (200IU in 0.05M phosphate buffer, pH 7.4) since this enzyme is more specific for collagen than is papain. All digestions were carried out at 37°C for 48 hrs. Following digestion, the samples were centrifuged for 10 mins at 9000rpm and the supernatant collected and stored frozen at -40°C

until required for assay.

2.4 Polyacrylamide gel electrophoresis

For the separation of corneal components that may have leached from the tissues into the storage medium, SDS-discontinuous polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Two types of study were performed to investigate firstly, any change in the amount of component leaching from the tissue into the medium as a function of tissue age and secondly, the amount of leaching as a function of period of storage of a tissue of known age in the incubation medium.

2.4.1 Sample preparation

Prior to electrophoresis 100 μ l volumes of each sample (study 1: volumes of incubation medium taken from storage of corneas of various ages, study 2 volumes of incubation medium taken from storage of particular corneas after 1 and 3 weeks in the same incubation medium) were heated in the presence of an excess of SDS and β -mercaptoethanol to ensure protein denaturation. Assuming that the resultant polypeptides bound SDS such that they possessed identical charge densities, migration in the polyacrylamide gels will have occurred according to polypeptide size (M_r). The SDS and mercaptoethanol were components of a sample buffer which consisted of 0.125M Tris (pH 6.75) containing 20% glycerol, 4% SDS, 10% β -mercaptoethanol and 0.002% bromophenol blue. This buffer was added to an equal volume of sample (i.e. 100 μ l) which was incubated for 5 mins at 100°C in a Techne Dri-block to dissociate the proteins by allowing the β -mercaptoethanol to reduce disulphide bonds.

2.4.2 Polyacrylamide gel preparation and running

Gels of dimensions 14 x 18cm and 1.5mm thickness were cast using an LKB 2001 vertical slab gel electrophoresis cell. Heated sample volumes of 50 μ l were loaded into preformed sample wells in the stacking gel. A discontinuous buffer system was prepared by polymerization of the resolving gel followed by polymerization of the stacking gel on top of this. The gels were generally run in pairs with an applied voltage of 150V (current 110mA) for approximately 5 hrs. In each case a 7.5% acrylamide gel was prepared for use as follows.

Resolving gel

7.5% acrylamide
0.2% bis-acrylamide
0.375M Tris-HCl pH 8.8
0.1% SDS
0.075% ammonium persulphate
33 μ l TEMED/100ml resolving gel

Stacking gel

3.5% acrylamide
0.1% bis-acrylamide
0.125M Tris-HCl pH 6.8
0.1% SDS
0.075% ammonium persulphate
75 μ l TEMED/100ml stacking gel

Molecular weight markers were run alongside the samples and contained the following proteins:

MW markers	M _r
Myosin	205,000
β -Galactosidase	116,000
Phosphorylase B	97,400
Albumin (bovine)	66,000
Albumin (egg)	45,000
Carbonic anhydrase	29,000

Following electrophoresis, the gels were carefully removed from the glass plates and fixed in a 10% TCA solution overnight and then stained for either protein or carbohydrate.

2.4.3 Staining of polyacrylamide gels

Coomassie blue protein stain

After fixing in TCA overnight, the gels were carefully placed in a solution of 0.125%(w/v) Coomassie blue in 10%(v/v) acetic acid/45%(v/v) methanol for approximately 5 hrs. The gels were then destained in several changes of 5%(v/v) methanol/7%(v/v) acetic acid until a clear background was obtained.

Alcian blue staining of glycosaminoglycans

The gels were fixed in 50%(v/v) methanol/7%(v/v) acetic acid for 1 hr and washed in distilled water for a further hour, these two steps were repeated since it was important to remove completely the SDS prior to gel staining. After washing, the gels were stained in a solution of 0.2%(w/v) Alcian blue/3%(v/v) acetic acid/0.2M MgCl₂ overnight. Following this, destaining in several changes of 3%(v/v) acetic acid/0.05M MgCl₂ was carried out until the gel background was clear. MgCl₂ was included in the staining and

destaining solutions to increase the specificity of staining and reduce background staining [Wall and Gyi, 1988].

The gels were photographed using Ilford Pan F ISO 50 black and white film. A Cannon A1 camera was used (with a Cannon FD lens 50mm) and Ilfospeed 5 photographic paper was used for good contrast prints.

2.5 Total glycosaminoglycan assay

The glycosaminoglycan content of the tissue culture medium was analysed using the microdetermination dye binding assay of Bartold and Page (1985). This method was chosen because of its sensitivity and its requirement for very little sample.

The assay relies upon the specificity of Alcian blue for glycosaminoglycans at an electrolyte concentration that minimizes the staining of other negatively-charged glycoproteins. Cellulose acetate electrophoresis membranes were marked at 2cm intervals along their length to make squares. A sample volume of 20 μ l (approximately 0.05-1.0mg ml⁻¹ GAG) was applied to each square and allowed to soak into the membrane. The strips were immersed in a staining solution of 0.2% Alcian blue in 0.05M magnesium chloride, 0.025M sodium acetate in 50%v/v ethanol/water. The strips remained in this solution for 30 mins at room temperature and were then destained in 3 changes (each of 15 mins length) of the same solution without the Alcian blue stain. Following destaining, the strips were blotted, cut at the marked 2cm intervals, placed in 2ml dimethyl sulfoxide and vigorously mixed until dissolved (approximately 15 mins). The absorbance of the resultant solution was read on a Pye Unicam SP6-400 UV spectrophotometer at a wavelength of 678nm. The absorbances were compared to those of a chondroitin sulphate standard curve (Figure 2.2).

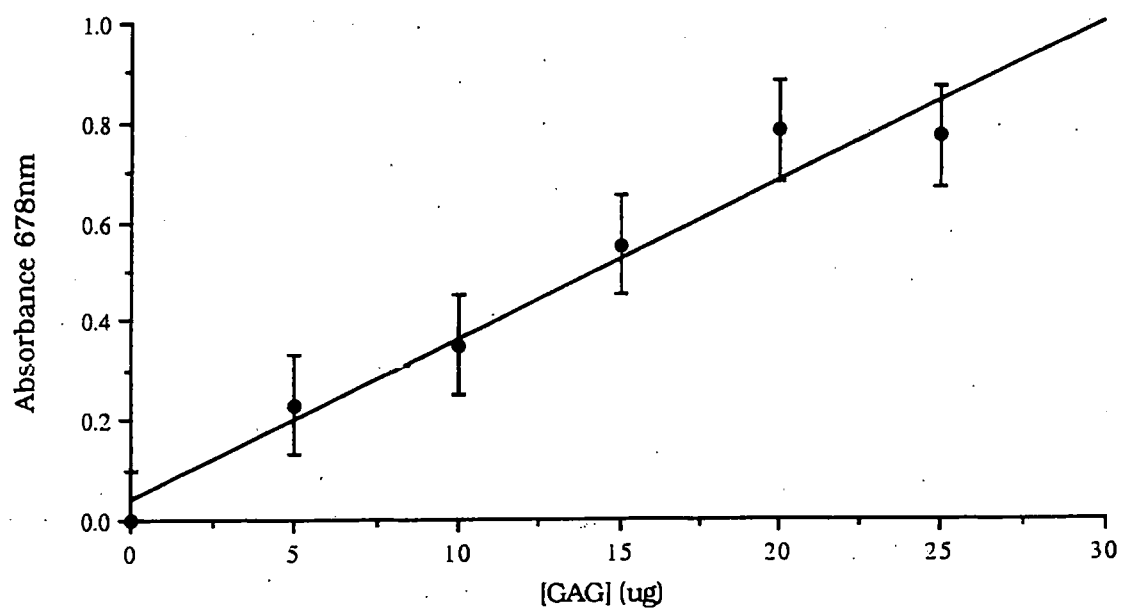


Figure 2.2 Standard curve produced using chondroitin sulphate standards for the total glycosaminoglycan assay

2.6 Hydroxyproline assay

In order to quantify the hydroxyproline content of both corneal and scleral collagen an assay devised by Woessner (1961) was employed. Prior to assay the corneal/scleral digests (0.2ml) were vortexed and acid hydrolysed with an equal volume of 12M hydrochloric acid in hydrolysis tubes. Hydrolysis was performed at 110°C for 18 hrs after which the digestion mixture was transferred to a graduated cylinder and washed three times with distilled water (1ml volume each time). A few drops of 10M sodium hydroxide were added to adjust the pH to within the range 5-7.5. The final volume of the solution was adjusted to 5ml with distilled water. Standards of known hydroxyproline concentration were prepared and run alongside these test samples using the procedure described below.

To 0.1ml of hydrolysed corneal/scleral sample was added 1ml of 0.05M chloramine T. The tubes were shaken and allowed to stand at room temperature for 20 mins. A 3.15M perchloric acid solution (1ml) was added, the tubes were again shaken and kept at room temperature for 5 mins. Finally 1ml of DMBA was added to each tube and after thorough mixing all tubes were placed in a water bath at 60°C for a further 20 mins. The absorbance of each solution was read using a Pye Unicam SP6-400 spectrophotometer at a wavelength of 557nm and the unknown hydroxyproline concentrations were read off the prepared standard curve (Figure 2.3).

2.7 Electron microscopy

Transmission electron microscopy was used on human corneas that had been incubated in culture medium for varying periods. By using a Cuproinic blue staining technique it was possible to investigate changes in tissue proteoglycan composition as a function of storage time.

The 'critical electrolyte' method of Scott and Orford (1981) was employed to stain the proteoglycans. Small pieces of cornea (approximately 1mm²)

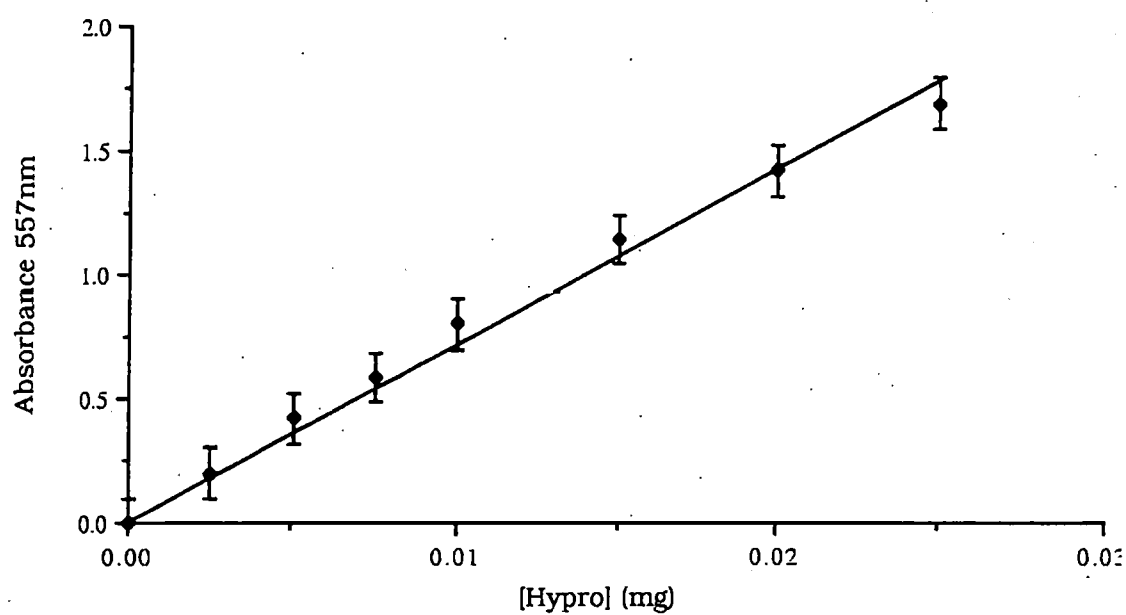


Figure 2.3 Standard curve for the hydroxyproline assay

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were stained overnight in 0.05% Cuprolinic blue in a 25mM sodium acetate buffer (pH 5.7) containing 2.5% glutaraldehyde and 0.1M $MgCl_2$. The following day the samples were rinsed for three 15 min periods in the buffer solution containing the fixative glutaraldehyde and $MgCl_2$. The samples were then washed three times (15 mins each time) with 0.5% aqueous sodium tungstate and then three times (again 15 mins each time) with 0.5% sodium tungstate in 50% ethanol. Following this the samples were put through an ethanol dehydration series and two 30 min changes in propylene oxide. The specimens were eventually placed in a 3:1 mixture of propylene oxide:Agar 100 resin overnight. The propylene oxide was allowed to evaporate out of the mixture since the sample bottles were left uncovered throughout this period. The samples were eventually placed in 100% Agar 100 resin for 12 hrs and then polymerized at 60°C for 48 hrs. From the embedded material silver-gold sections of approximately 70nm thickness were cut using a Dupont diamond knife on a Reichert-Jung Ultracut E ultramicrotome.

Chapter 3

Development of Glycation Procedures

Assays for the glycation of collagen are limited as a result of the interference by the natural cross-links of collagen and enzymically-linked sugar (glycosylation) where the glycosidic bonds have been destroyed. The assays often quantify the carbonyl moiety of the glycated protein, commonly the SB and AP. The AP from the natural cross-links of collagen can affect the results of such assays unless reduced with sodium borohydride prior to glycation. The thiobarbituric acid assay (TBA) is a widely used colorimetric method for measuring protein glycation specifically the amount of AP. Since it is a time-consuming procedure it has been largely discontinued by routine laboratories but it has been used successfully to measure glycated collagen. Even though the TBA method has been superseded by other methods, it remains a simple, convenient and inexpensive method for assaying glycated collagen.

3.1 Thiobarbituric acid (TBA) assay

A microplate version of the TBA method by Parker et al (1981) was developed here to measure relative amounts of glycation in both corneal and scleral collagen across the entire age range of samples and the assay

was also used for in vitro glycation assessments. Essentially the assay works by converting the sugar moiety of the glycosylated protein to 5-hydroxymethylfurfural (5-HMF; illustrated in Figure 3.1) by heating with oxalic acid. The adduct formed by reacting 2-thiobarbituric acid with 5-HMF is measured photometrically and results are expressed as nanomoles of HMF or fructose equivalents. A fructose standard curve is used because the sugar moiety of a glucose AP has the fructose configuration, i.e. the carbonyl group is located on the C-2 position of the AP. The absorbance of the coloured adduct is proportional to the concentration of Amadori product in the protein. This method has the advantage that the Schiff bases and collagen cross-links do not interfere with the assay [Furth, 1988]. Mild hydrolysis of 0.1ml of sample (supernatant obtained from collagen digestion) with 0.1ml of 0.5M oxalic acid was performed at 124KPa, 124°C for 1 hr to release 5-HMF. The protein was precipitated by addition of 0.1ml 40% TCA. Following centrifugation at 10,000rpm for 10 mins, 0.2ml of supernatant was removed and transferred to a microplate. To the supernatant was added 0.067ml of 0.05M TBA (brought to pH 6 with NaOH). The microplate was incubated at 37°C for 35 mins after which the absorbance was read at 450nm on a Bio-Tek microplate autoreader. Fructose standards were used to produce a standard curve (Figure 3.2) from which the 5-HMF concentration (nmols in 0.1ml of sample) could be read for both corneal and scleral digests. A reagent blank (0.1ml of 0.15M saline) was prepared and run alongside the test samples and standards. During this thesis, the method of Parker et al was further modified and adapted for use with a microplate reader. This modified version has an improved yield of HMF and increased sensitivity requiring only 1 mg of protein. Using the microplate autoreader it is also possible to read up to 96 samples in 40 seconds.

Free sugars do however interfere in the assay but can be readily removed by dialysis. It has been suggested by Schleicher et al (1981) that enzymically bound sugars interfere in the TBA method since any 6-carbon sugar (or that containing greater than 6-carbon atoms) whose AP has a carbonyl

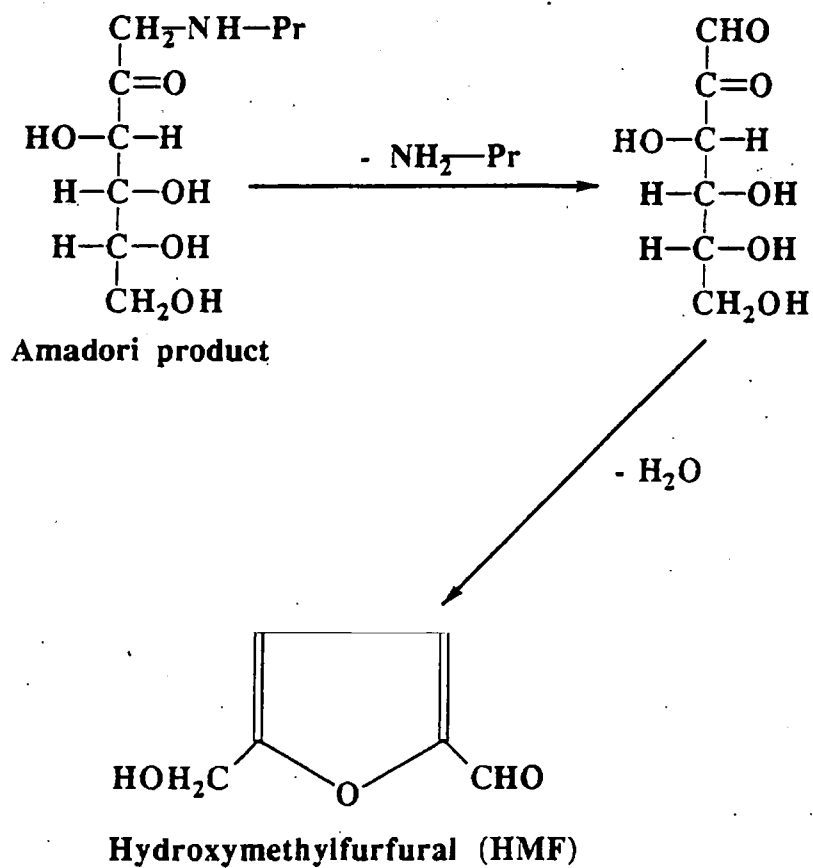


Figure 3.1 Formation of 5-hydroxymethylfurfural (HMF) during the TBA assay

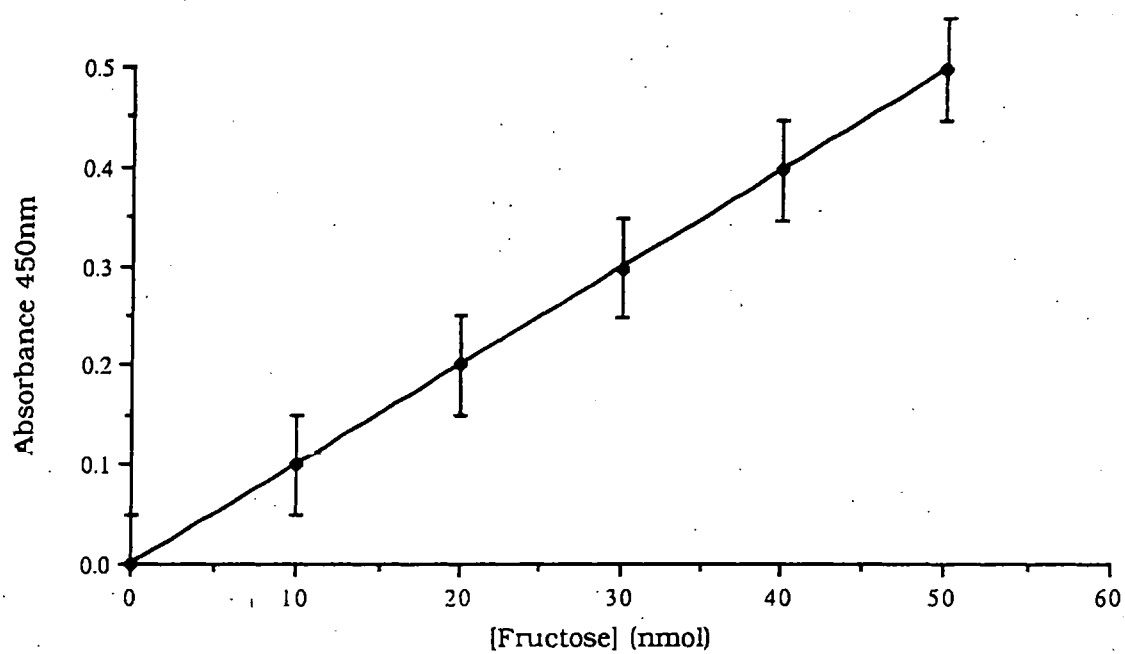


Figure 3.2 Standard curve produced by using fructose standards for the thiobarbituric acid assay

group on the C-2 position, could result in ring-closure to form 5-HMF. The corneal and scleral proteoglycans are a source of 6-carbon sugars however, these are removed by prolonged extraction in guanidine hydrochloride (described earlier) prior to the TBA assay. Using in vitro glycated collagen as a model glycoprotein, the investigation performed below suggests that the total interference by enzymically bound sugar is <8%.

TBA assay - Interference by glycoproteins

The in vivo formation of the reducible collagen cross-link dehydrodihydroxylysinoxynorleucine undergoes a spontaneous Amadori rearrangement to form hydroxylysino-5-ketonorleucine (Figure 3.3). This structure, an Amadori product, may well interfere in the TBA assay such that the TBA assay value becomes a measure of AP from both glycation of collagen and the formation of reducible cross-links in collagen with age. Borohydride reduction of in vitro glycated type I collagen (from bovine tendon) was performed by incubating with 55mM sodium borohydride in 1mM NaOH, pH 7.4 at 37°C overnight [Olufemi et al., 1987]. The incubation mixture was placed on a magnetic stirrer throughout this period. At the same time, another aliquot of in vitro glycated type I collagen (10mg/ml) was incubated in the absence of sodium borohydride i.e. in 1mM NaOH, pH 7.4 at 37°C. The reduction was terminated by reducing the pH to 3.0 using concentrated acetic acid and the collagen was dialysed exhaustively against water to remove any unreacted borohydride. Following this, both collagen samples were centrifuged at 9000rpm for 10 min and the precipitate of each sample was resuspended in 0.05M phosphate buffer, pH 7.4, containing 200 IU collagenase and incubated at 37°C for 48 hrs. Chloroform and toluene (5 μ l each) were added to prevent bacterial growth. After centrifugation at 9000rpm for 10 min, the supernatant for each sample was collected and used for TBA analysis. Type I collagen was also extracted from human corneas and scleras (using approximately 5-10mg of tissue) after removing the tissue proteoglycans. The extracted samples were also subjected to borohydride reduction as above and were digested with papain (0.5mg/ml citrate buffer, pH 6) at

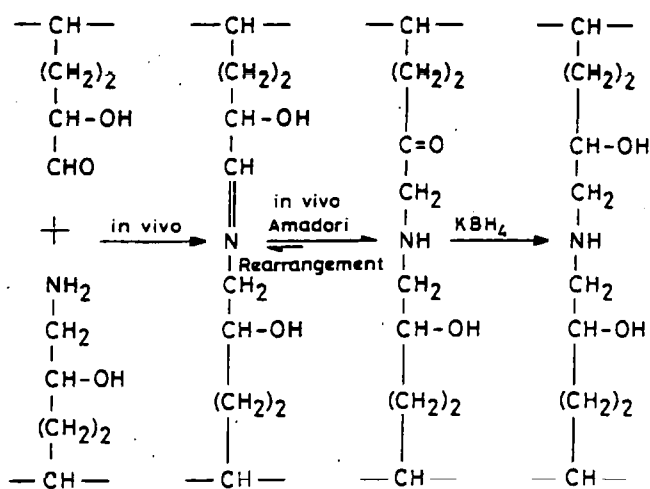


Figure 3.3 The formation in vivo of the reducible cross-link dehydrodihydroxylysino-5-ketonorleucine, its spontaneous Amadori rearrangement to form the keto-imine hydroxylysino-5-ketonorleucine, and its reduction in vitro to form dihydroxylysino-5-ketonorleucine

50-55°C for 24 hr prior to TBA analysis.

In the absence of borohydride reduction of type I collagen, the amount of HMF generated was 0.012 nmol/nmol hypro. Following borohydride reduction this figure fell to 0.011 nmol/nmol hypro. This implies that an interference of approximately 8% is occurring and it may be possible to use this procedure to correct for interference by glycoproteins.

TBA assay - Yield of chromophore

Part of the investigation looked at percentage of HMF lost during the heating step in the autoclave method. The importance lies in the need to balance the production of HMF with its destruction. Parker et al had reported 30% loss of HMF after 1 hr of heating in an autoclave whereas the microplate version, by comparing the quantity of chromophore produced by prepared HMF standards when mixed either directly with TBA or subjected to the full assay procedure, found only a 13% loss of HMF. The autoclave version of the TBA method introduced by Parker et al has the advantage of requiring only 1 hr of hydrolysis compared to earlier versions which needed between 4 and 8 hrs (Ney et al., 1981; McFarland et al., 1979) and hence resulted in greater loss of HMF.

The in vivo glycation of human corneal and scleral collagen as measured by this microplate version of the TBA assay, produced values of 9.24 ± 0.235 and 1.50 ± 0.245 nmol HMF/mg collagen respectively for tissue ages between 20-30 years. Since human skin collagen is known to be more glycated than both corneal and scleral collagen, the results of this investigation compare well with those of other workers who have employed the autoclave version of the TBA method - they obtained a value of 14.58 ± 3.11 nmol HMF/mg collagen for tissues aged between 20-30 years [Miksik and Deyl, 1991].

3.2 Fluorescence emission measurements for AGEs

Structural changes in glycated proteins can be explored by measuring changes in fluorescence which are believed to indicate cross-linking and AGE formation in a variety of proteins (Sell and Monnier, 1989b; Ahmed and Furth, 1990). One fluorescent cross-link structure which has been relatively well characterised is pentosidine (described in Chapter 1). Its optimum wavelengths are 325nm (excitation) and 375nm (emission) and it is considered to be the major fluorophore in glycated model proteins [Dyer et al., 1991]. However, for glycated collagen the excitation-emission wavelengths that give maximum fluorescence are 370nm and 440nm respectively (Kohn et al., 1984; Monnier et al., 1986; Miksik and Deyl, 1991; Odetti et al., 1992). Even though pentosidine is found in collagen in vivo [Sell and Monnier, 1989], the wavelengths most sensitive to glycation-induced change were used here.

Fluorescence emission of the corneal and scleral digests was measured using an Aminco-Bowman spectrophotofluorometer (model J4-8960). Approximately 1ml of supernatant was required for the glass cuvettes used and the machine was zeroed on the appropriate enzyme blank (either papain or collagenase). Most measurements were made at an excitation wavelength of 370nm with emission at 440nm [Kohn et al, 1984] and results were expressed as arbitrary units of fluorescence per mg of hydroxyproline and in some cases as units per mg dry weight tissue.

3.3 In vitro glycation of collagen

Tissue (both corneal and scleral) and collagens of known weights (types I and IV from Sigma Chemical Company) were incubated in 0.5M glucose, 0.05M phosphate buffered saline, pH 7.4 and 3mM sodium azide at a temperature of 37°C for specified time periods. (Studies were also

performed with 0.5M fructose and 0.5M ribose). The pH of the incubations was checked occasionally using a Schott Gerate pH meter (Model no. CG 727) to ensure no change. The samples at glycation point 'zero' were incubated in buffer/azide for the entire period of the experiment. Following each specified time point, the samples were dialysed to remove any unreacted sugar incubated in fresh buffer for the remainder of the study in order to ensure that the changes observed in X-ray diffraction were not simply effects of alterations in tissue hydration brought about by prolonged incubation in the buffer at 37°C.

3.3.1 Dialysis of glycated collagen

Dialysis was performed to allow removal of free sugars following in vitro glycation. These may have otherwise interfered in the biochemical assay for glycation. Dialysis tubing with a molecular weight cut-off of 12-14 kDa was prepared by soaking in 1% acetic acid solution for 1 hr after which it was rinsed for approximately 30 mins in distilled water. The tubing was further soaked for 30 mins in a solution of 1% sodium carbonate with 10mM EDTA. The tubing was heated in this solution to 75°C. After heating in distilled water, the tubing was stored at 4°C in distilled water before use. Dialysis was performed at 4°C in approximately 2L of distilled water with several changes overnight.

3.4 Inhibition of glycation and AGE formation

Three compounds were chosen to investigate their effects on the in vitro glycation of corneal collagen. Corneal tissue (from 3 donors of similar age) was carefully sectioned and incubated at 37°C in 0.05M phosphate buffer (pH 7.4) with 0.5M fructose, 3mM sodium azide and an inhibiting compound, either 25mM acetylsalicylic acid (aspirin; ASP), 25mM

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diethylenetriaminepentaacetic acid (DETAPAC) or 25mM aminoguanidine (AG) for a period of 24 days in total. Control samples were incubated in the absence of fructose. At each time point (3, 6, 15 and 24 days) the tissue sections were dialysed overnight against distilled water to remove traces of sugar and inhibitor and the sections were reincubated in fresh phosphate buffer with sodium azide for the remaining period (to 24 days). Each tissue was examined using the high angle camera at the synchrotron radiation source at Daresbury.

Chapter 4

Corneal Incubation Medium - Results and Discussion

Leaching of proteoglycans and soluble proteins occurs when denuded corneas are placed in a bathing medium [Hughes, 1983]. The corneas used for the studies in this thesis were supplied as corneoscleral discs which had been stored in an incubation medium for up to 30 days at 34°C before investigation was possible; hence it was necessary to ensure that significant loss of protein or proteoglycan was not occurring from the tissue to the culture medium during this period. Although the CTS Eye Bank do not use corneas that have been stored in medium for such periods of time, it was of interest to investigate the efficiency of the medium during this period.

4.1 SDS-polyacrylamide gel electrophoresis

This technique was employed to investigate protein loss from the culture medium. Essentially two types of experiment were performed :

1. an age-related study to investigate whether there was any relationship between the age of the cornea and the amount of protein lost
2. a time-related study to investigate the correlation between the period of storage and the amount of protein lost.

Polyacrylamide gels were stained for protein and proteoglycan. However,

using Alcian blue, proteoglycan staining was extremely faint hence a more sensitive assay was used, the results of which are presented in section 4.2. The results of the protein staining are given in Figures 4.1 and 4.2. Protein bands were seen at 45-66k, 66k and just above 66k (Fig 4.1, using a low molecular weight marker). From Fig 4.2, where a high molecular weight marker was used, the larger protein band was approximately 76k in size.

4.2 Total glycosaminoglycan assay

A dye binding assay was used to measure the concentration of GAG within the culture medium. The method relies on the specificity of Alcian blue for GAG at a critical electrolyte concentration which minimises the staining of other negatively-charged glycoproteins. The assay was performed as a function of age and as a function of time, the results are presented in Table 4.1 and Table 4.2.

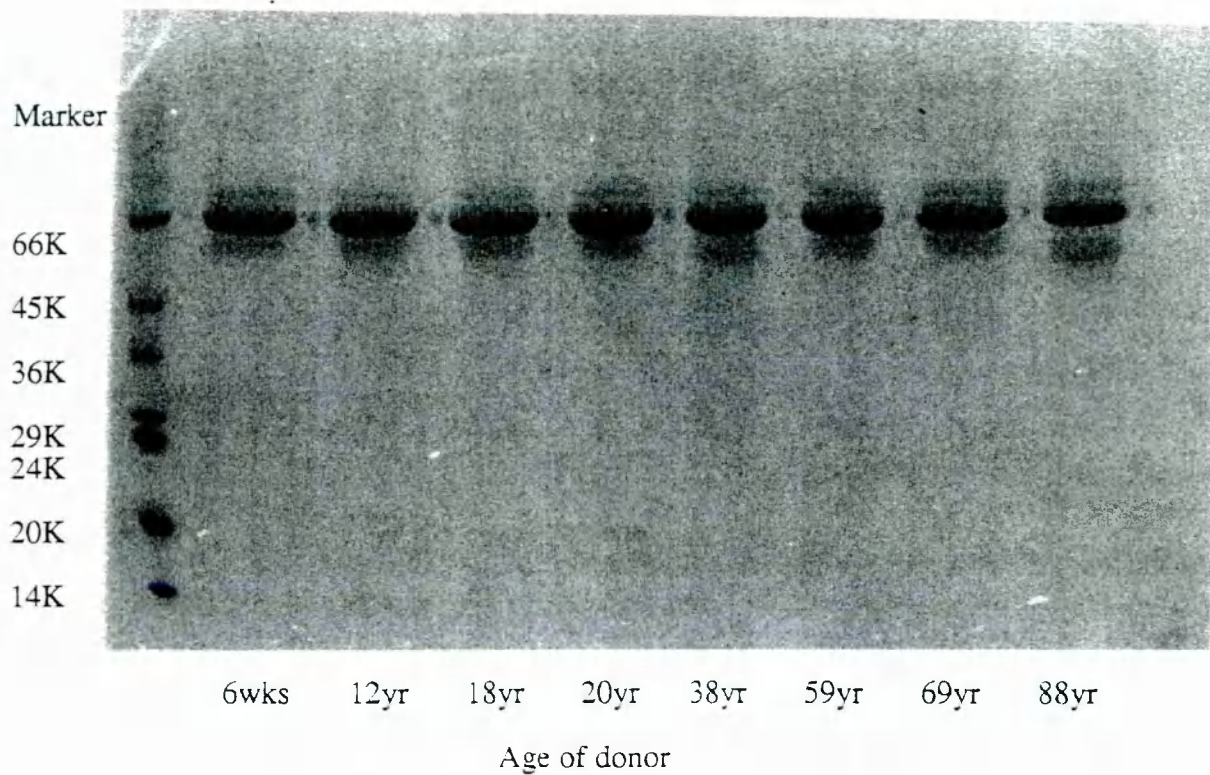


Figure 4.1 Age-related study : 7.5% polyacrylamide gel stained with Coomassie blue for protein from corneal culture medium. Medium taken from donor corneas of 6 weeks-88 years of age

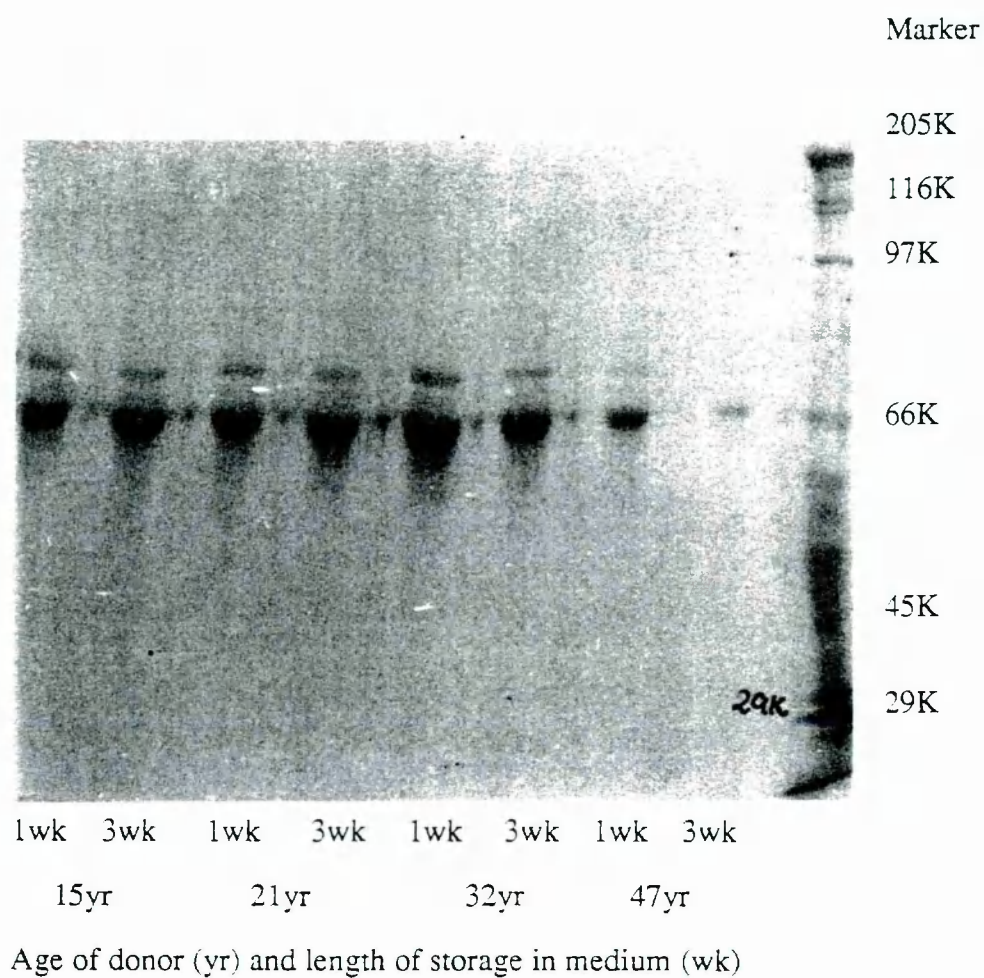


Figure 4.2 Time-related study : 7.5% polyacrylamide gel stained with Coomassie blue for protein from corneal culture medium of 4 corneas (ages 15, 21, 32 and 47 years) after storage periods of 1 and 3 weeks

4.3 Electron microscopy

Cultured corneas, both young and old, were examined by electron microscopy. In particular, the staining of proteoglycans within these samples was compared with that of an uncultured cornea. The results are presented in Figures 4.3, 4.4, 4.5 and 4.6.

4.4 Discussion of incubation medium studies

Polyacrylamide gel electrophoresis suggests that proteins are lost from corneas of all ages to the corneal storage medium (Fig 4.1), as indicated by Coomassie blue staining. The 66k protein band is the most heavily stained and may well be albumin, however the incubation medium itself was investigated using SDS-PAGE and found to produce a heavily stained band at 66k. It is very likely that the foetal calf serum component of the culture medium is responsible for this band. A diffuse staining between 45-66k could represent a 54k protein described in bovine cornea. In all cases the loss of protein at bands 54k and 76k was unrelated to the age of the cornea that had been stored in that medium.

Since the corneas used in the X-ray diffraction and glycation studies had already been stored in the medium, in some cases for several weeks, it was necessary to investigate whether the period of storage was affecting the concentration of proteins (and proteoglycans for that matter) and in particular it was of interest to pin-point at what period of storage losses became important. From Figure 4.2, a heavily-stained protein band again appeared at 66k and was assumed to be the foetal calf serum component of the culture medium. A protein band also appeared between 66k and 97k although this was not as apparent in the medium taken from a 47 year old cornea. In this study medium was taken from 4 corneas (age 15-47 years) at two time points - 1 and 3 weeks. With the exception of the medium

Age of stored cornea from which medium was analysed (years)	Relative GAG concentration ($\mu\text{g ml}^{-1}$)
4	6.43 \pm 0.28
26	0.98 \pm 0.11
35	2.50 \pm 0.21
43	14.10 \pm 0.15
50	10.00 \pm 0.19
62	3.65 \pm 0.21
75	12.18 \pm 0.25
89	2.88 \pm 0.23

Table 4.1 The level of GAG in the corneal storage medium of corneas aged from 4 to 89 years at a storage period of 3 weeks

Corneal storage medium	Time at which aliquot of medium taken (wks)	Relative GAG concentration ($\mu\text{g ml}^{-1}$)
From 15 yr old cornea	1	0.98 +/- 0.15
	3	10.0 +/- 0.20
From 53 yr old cornea	1	0.52 +/- 0.15
	3	0.21 +/- 0.17
	5	0.31 +/- 0.15
From 95 yr old cornea	1	0.61 +/- 0.16
	3	14.42 +/- 0.20
	5	14.10 +/- 0.18

Table 4.2 The level of GAG in the corneal storage medium of 3 corneas at 1, 3 and 5 weeks

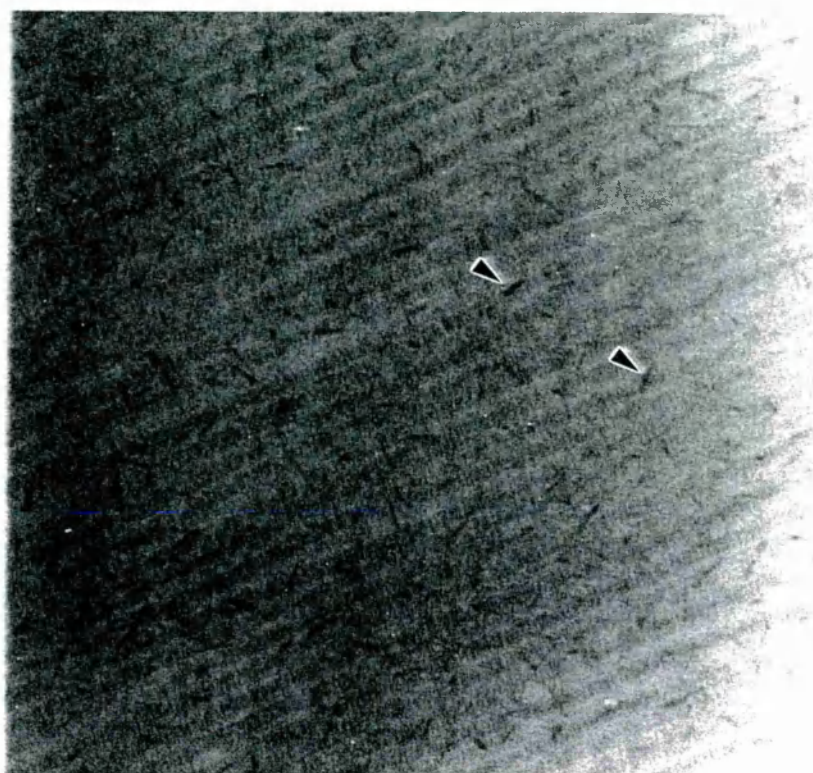


Figure 4.3 Electron micrograph showing cross-section through a Cuproline blue stained 37 year old uncultured human cornea, counterstained for collagen with uranyl acetate. The proteoglycans are indicated by arrows. Magnification 86,000 (Electron micrograph courtesy of I.M.Rawe)

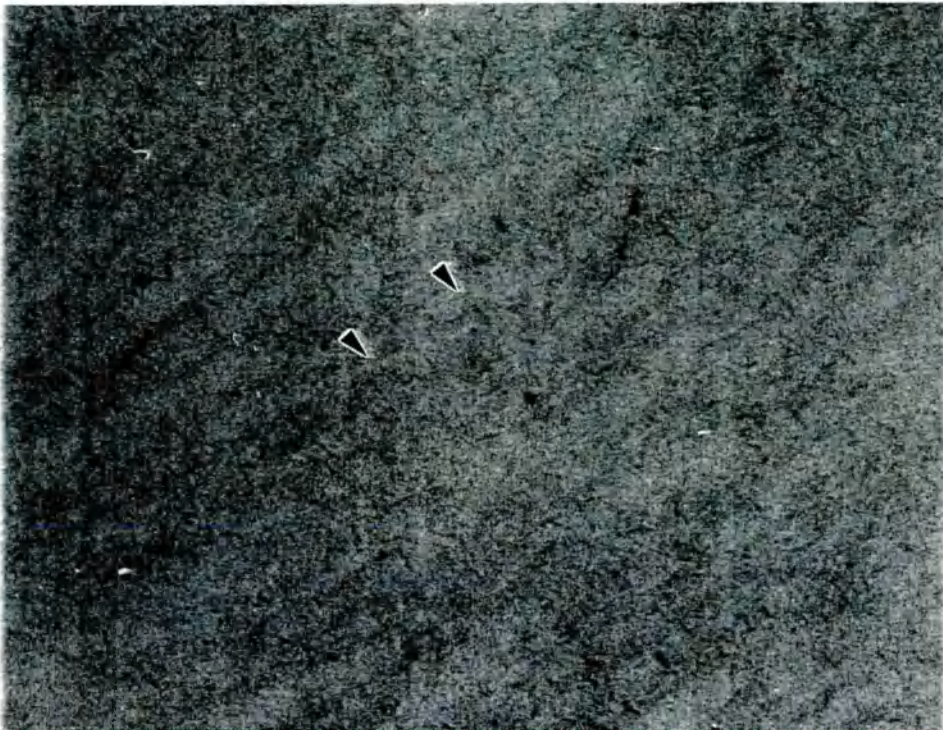


Figure 4.4 Electron micrograph showing cross-section through a Cuproline blue stained 27 year old cultured human cornea. The proteoglycans are indicated by arrows. Magnification 86,000



Figure 4.5 Electron micrograph showing cross-section through a Cuproinic blue stained 43 year old cultured human cornea. The proteoglycans are indicated by arrows. Magnification 86,000

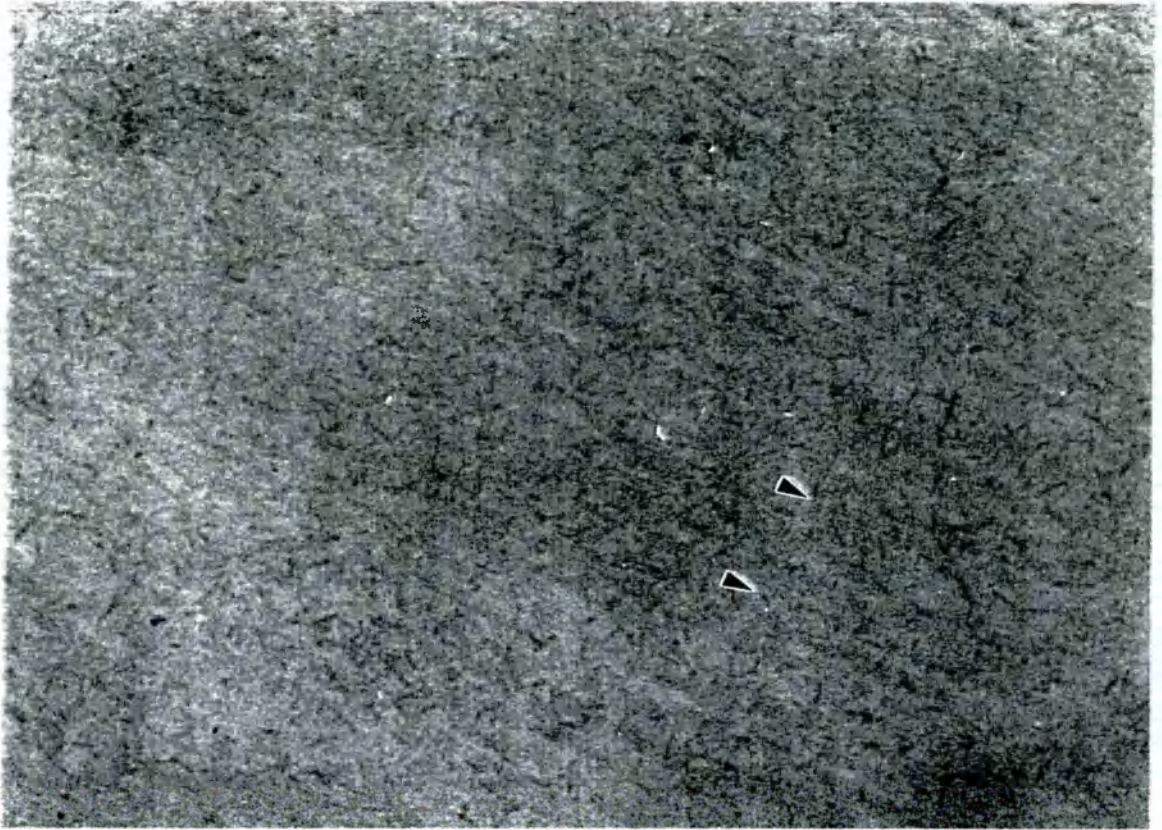


Figure 4.6 Electron micrograph showing cross-section through a Cuproline blue stained 93 year old human cultured cornea. The proteoglycans are indicated by arrows. Magnification 86,000

housing the 47 year old sample, the intensity of the protein bands remained unchanged from 1 to 3 weeks. Although there were losses of proteins from the tissue, most were unrelated to the age of the tissue. However, the amount of the 66-97k protein lost appears to decrease with age. All tissues that were eventually used for X-ray diffraction studies were selected such that they had been stored in the culture medium for equal periods.

Polyacrylamide gel electrophoresis can also be used to look at the leaching of tissue proteoglycans. It simply involves using an Alcian blue stain for acidic or sulphated groups on GAGs [Wall and Gyi, 1988]. However, the lack of Alcian blue staining with PAGE implied no significant losses of proteoglycan to the storage medium, hence a more sensitive technique was employed - a microdetermination dye binding assay for GAGs. Table 4.1 shows that proteoglycan loss is occurring from corneas across the entire age range, but there appears to be no relationship between its concentration at a specified time point and the age of the cornea stored in the medium.

Table 4.2 shows that proteoglycan loss to the storage medium is occurring from both the young and older corneas. In the cases of the 15 and 95 year old samples, the concentration of proteoglycan in the storage medium increases from $0.98\text{-}10.0\ \mu\text{g ml}^{-1}$ and $0.61\text{-}14.10\ \mu\text{g ml}^{-1}$ respectively from 1 to 3 weeks storage.

Figures 4.4, 4.5 and 4.6 demonstrate through Cuproline blue staining of a 27 year old, 43 year old and 93 year old human cornea, that proteoglycans are still present within the corneal stroma and when compared with the electron micrograph of an uncultured human cornea given in Figure 4.3, the loss to the medium appears to be small. There also appears to be no significant difference in the proteoglycan content when comparing the three cultured corneas.

By binding to specific bands on the collagen fibrils, proteoglycans affect the regularity and the parallel spacing of collagen fibrils, and thus contribute to the transparency of the cornea. The loss of proteoglycans during organ culture was small and it is likely that the intact corneal epithelial and endothelial cell layers minimised this loss since Slack et al (1992) have

shown that removal of these layers results in a three to fivefold greater loss. It has also been shown that, on removal of the corneal epithelium, glycoprotein loss is also increased. It is probable that the scleral rim and the intact epithelium and endothelium prevent leaching of proteoglycans and other constituents of the stroma. Hodson et al (1991) have shown that components of M_r 10 kDa cannot penetrate the epithelial cell layer or Descemet's membrane. The results of Slack et al show that, with both the endothelial and epithelial cell layers intact, only a 1% loss of proteoglycan occurs during corneal preservation (less than 2% after 14 days storage). These studies thus support the results of the corneal medium investigation in this thesis. It was therefore possible to use cultured corneas for the experiments described in the following chapters.

Chapter 5

X-Ray Diffraction and Glycation of Tissue - Results and Discussion

This chapter will describe the results obtained from X-ray diffraction of the corneal and scleral tissues received from the CTS Eye Bank. In an attempt to account for the results obtained, the levels of glycation of chosen corneas and scleras were also investigated along with levels of fluorescence. The two parameters of interest using X-ray diffraction were the spacing between adjacent collagen molecules within the fibrils, and the centre-to-centre spacing between the fibrils themselves. However, when either of these spacings changes, the molecular or fibrillar network expands or contracts in *two* dimensions. Hence the parameters of interest are 1) the (molecular spacing)² which is referred to as the intermolecular unit cell and 2) the (interfibrillar spacing)², which is referred to as the fibril unit cell. All studies were carried out as a function of tissue age.

5.1 X-ray diffraction

Three studies were carried out, one in which corneal hydration was not set prior to analysis (Figures 5.1 and 5.2), a second in which it was set with polyethylene glycol to a value just below physiological (Figures 5.3 and 5.4), and a third study where corneal and scleral hydrations were set as

close to physiological as possible (Figures 5.5, 5.6 and 5.7). Although there is no reason to presume that the increase in the intermolecular unit cell of collagen is linear, the line of 'best fit' is plotted to indicate the trend in the data points.

5.2 Glycation

The levels of in vivo glycation of both corneal and scleral collagen (Figures 5.8 and 5.9), were investigated by using the TBA assay. Fluorescence (370nm/440nm) was also read for both tissues (Figures 5.10 and 5.11).

5.3 Discussion

Figures 5.1 and 5.2 illustrate respectively the changes in the collagen molecular unit cell and fibril unit cell within human corneas as a function of age. The intermolecular unit cell appears to increase with tissue age, however the increase is small with a correlation coefficient, r , of 0.43. Since the corneal hydrations were not set, corneal hydrations were found to vary between 1.8 and 5.0. However, this represents a true age effect because the size of the intermolecular unit cell does not vary much above $H=2.5$ [Meek et al., 1991]. If this growth is linear it corresponds to a 7% increase from $3.28 \pm 0.07\text{nm}^2$ (birth) to $3.52 \pm 0.12\text{nm}^2$ (age 90). In Figure 5.2 the interfibrillar unit cell shows no apparent correlation with tissue age. In this case, the tissue hydration is probably responsible for the scatter of the data, since the interfibrillar spacing is very hydration dependent (Sayers et al., 1982; Meek et al., 1991). In order to assess to what extent the observations in Figures 5.1 and 5.2 were due to variations in hydration, the experiments were repeated, this time setting the tissue hydration beforehand. As a starting point, 2.5% PEG was used, since this had been found to produce a physiological level of hydration in bovine corneas [Meek et al., 1991].

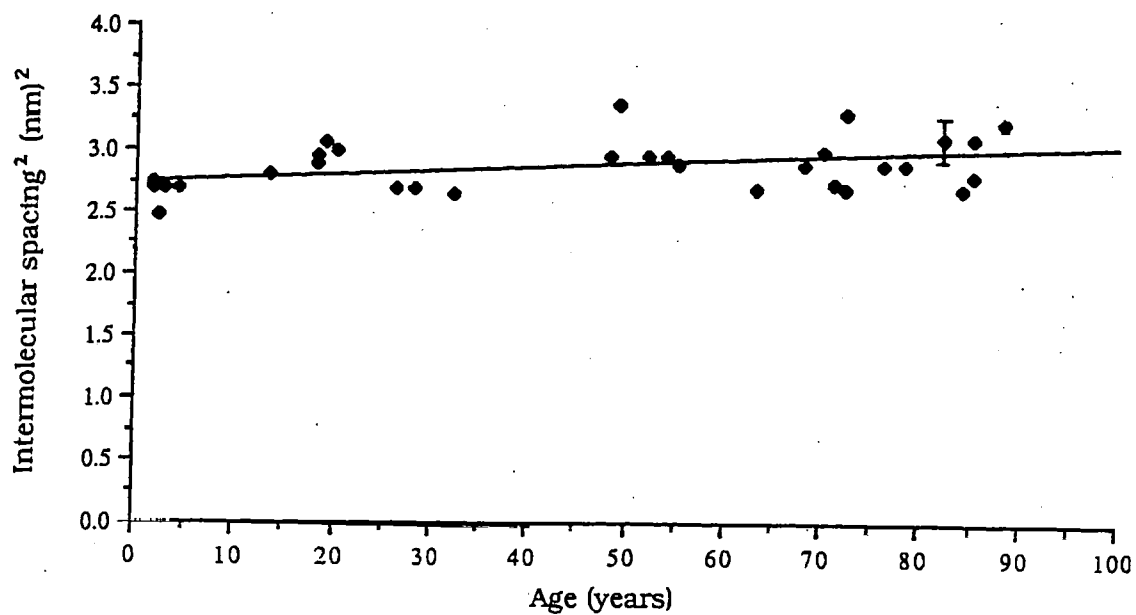


Figure 5.1 Change in (intermolecular spacing)² of human corneal collagen with age (hydration not set, hence range of hydrations 2.2-4.5)

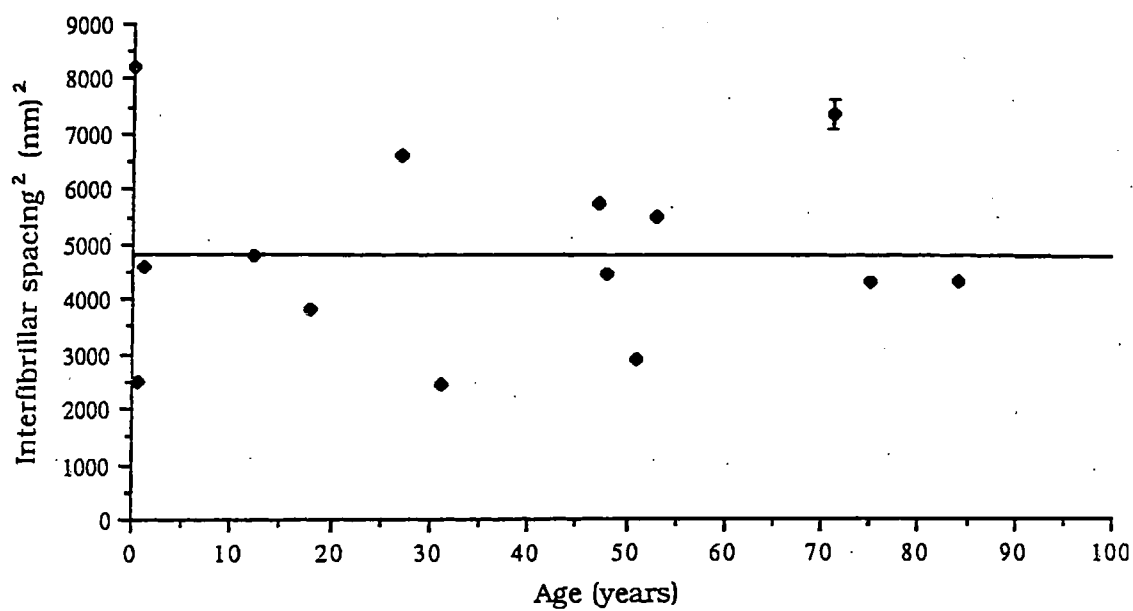


Figure 5.2 Change in (interfibrillar spacing)² of human corneal collagen with age (hydration not set, hence range of hydrations 2.2-4.5)

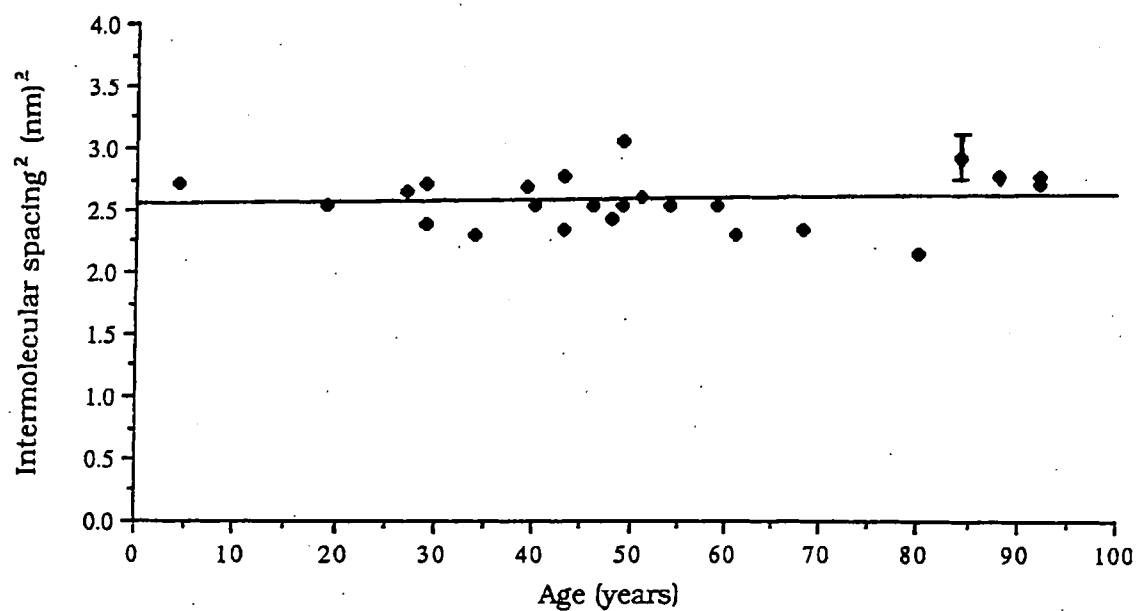


Figure 5.3 Change in (intermolecular spacing)² of human corneal collagen with age (hydration set by equilibrating with 2% PEG (H=2.5))

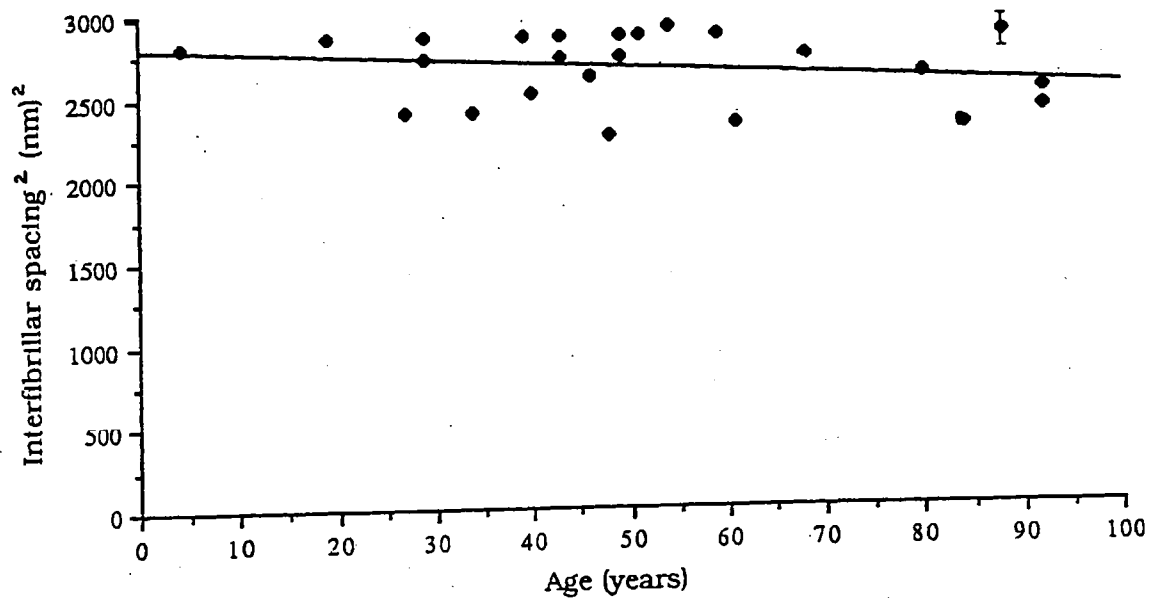


Figure 5.4 Change in (interfibrillar spacing)² of human corneal collagen with age (hydration set by equilibrating with 2% PEG (H=2.5))

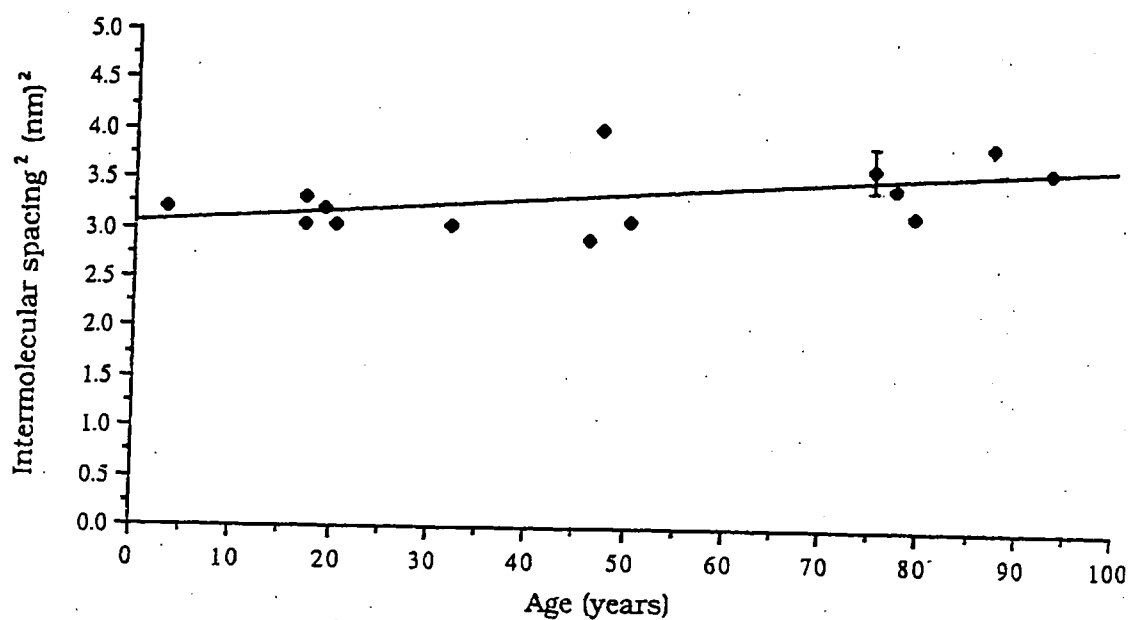


Figure 5.5 Change in (intermolecular spacing)² of human corneal collagen with age($H=3.4$)

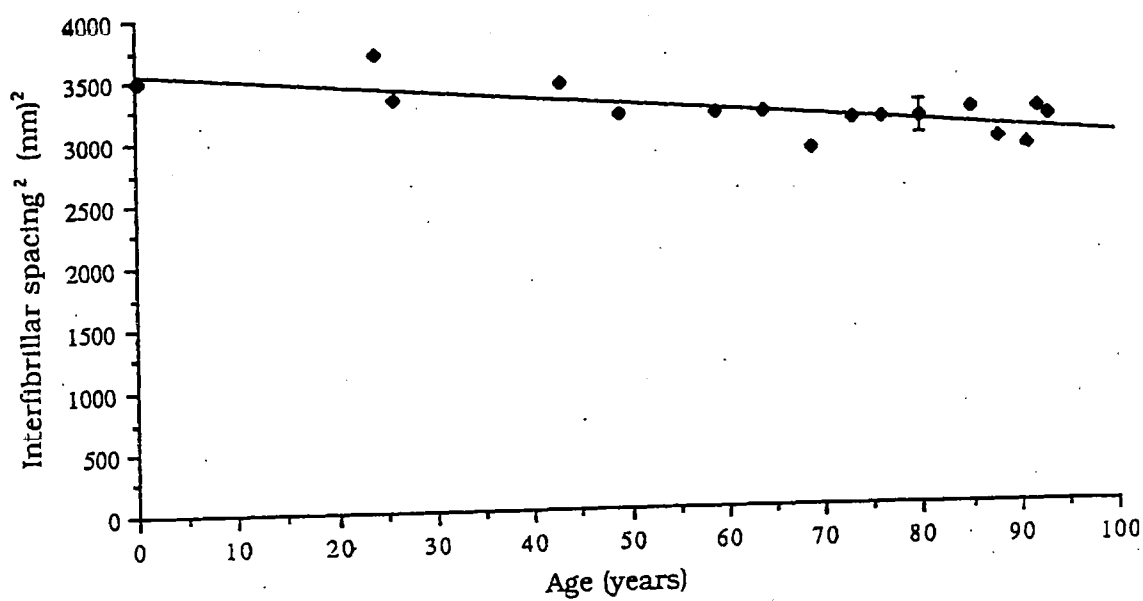


Figure 5.6 Change in (interfibrillar spacing)² of human corneal collagen with age ($H=3.4$)

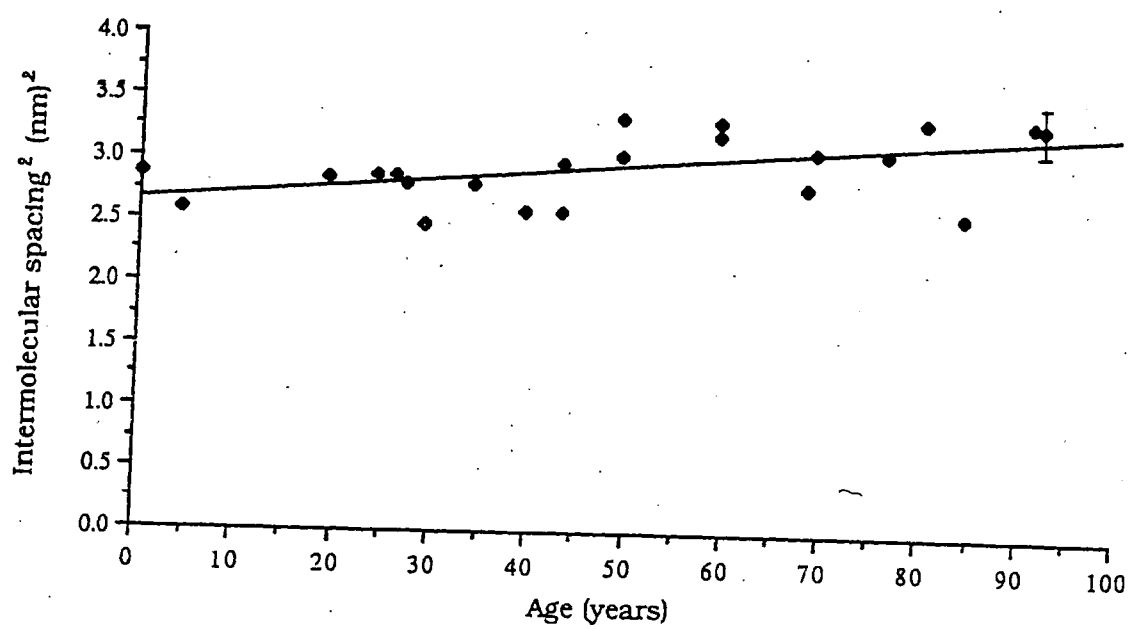


Figure 5.7 Change in (intermolecular spacing)² of human scleral collagen with age ($H=3.3$)

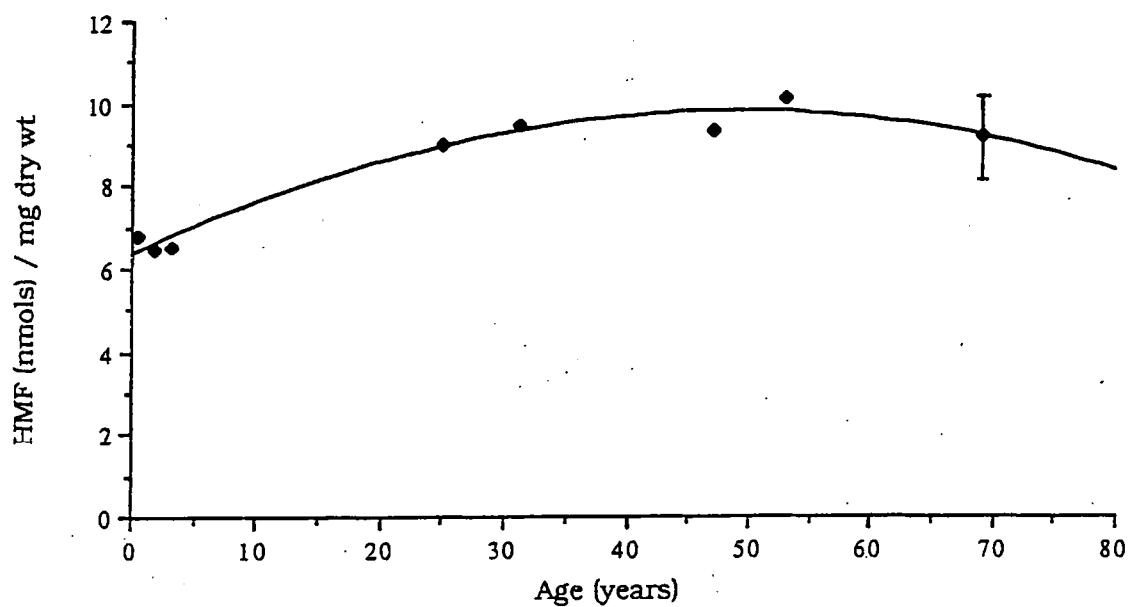


Figure 5.8 Change in glycation of corneal collagen with age. An initial increase is seen in corneal glycation but, beyond about 50 years of age, the curve appears to level off

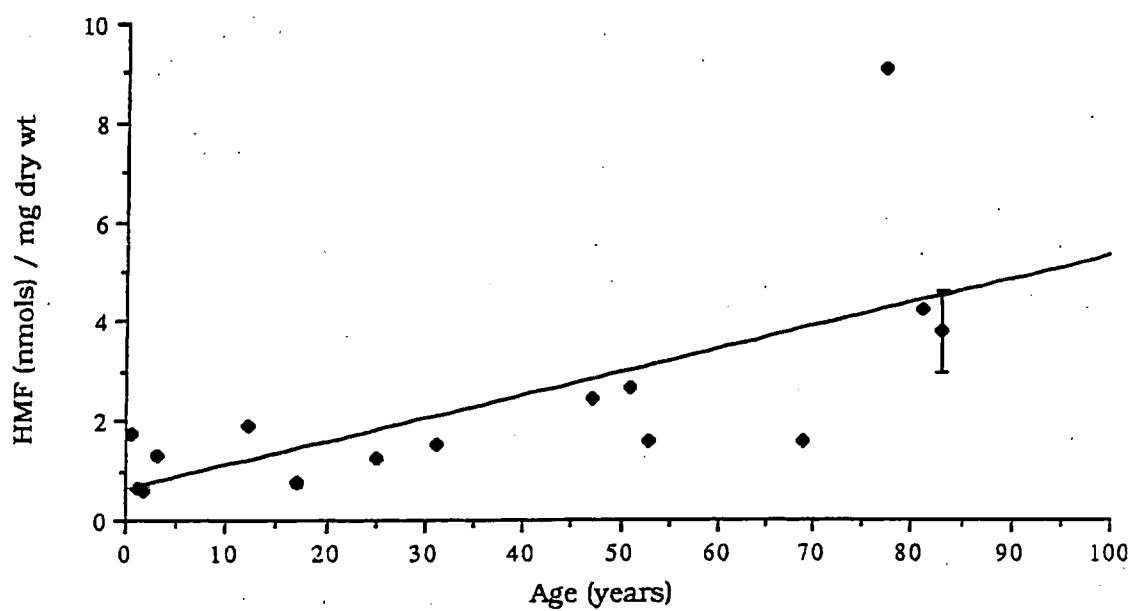


Figure 5.9 Change in glycation of scleral collagen with age. A linear increase is observed in the concentration of HMF in scleral collagen ($r=0.68$)

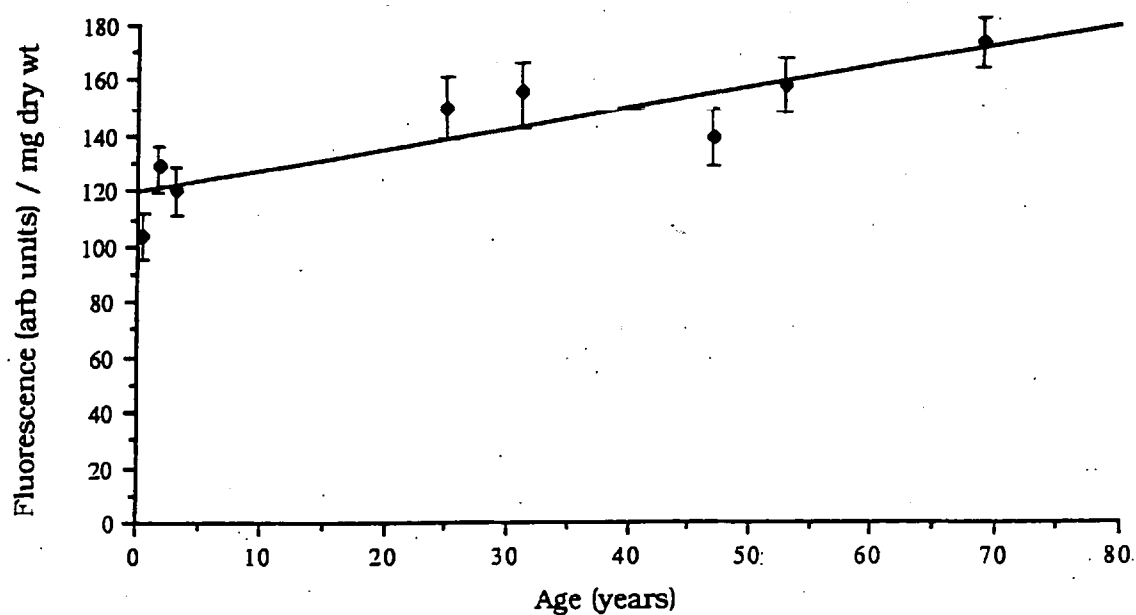


Figure 5.10 Change in fluorescence of corneal collagen with age. An increase in fluorescence is observed, but it is unclear whether the increase is linear and whether it continues beyond the age of about 30 years. The line of 'best fit' is plotted to indicate the upward trend in the data points

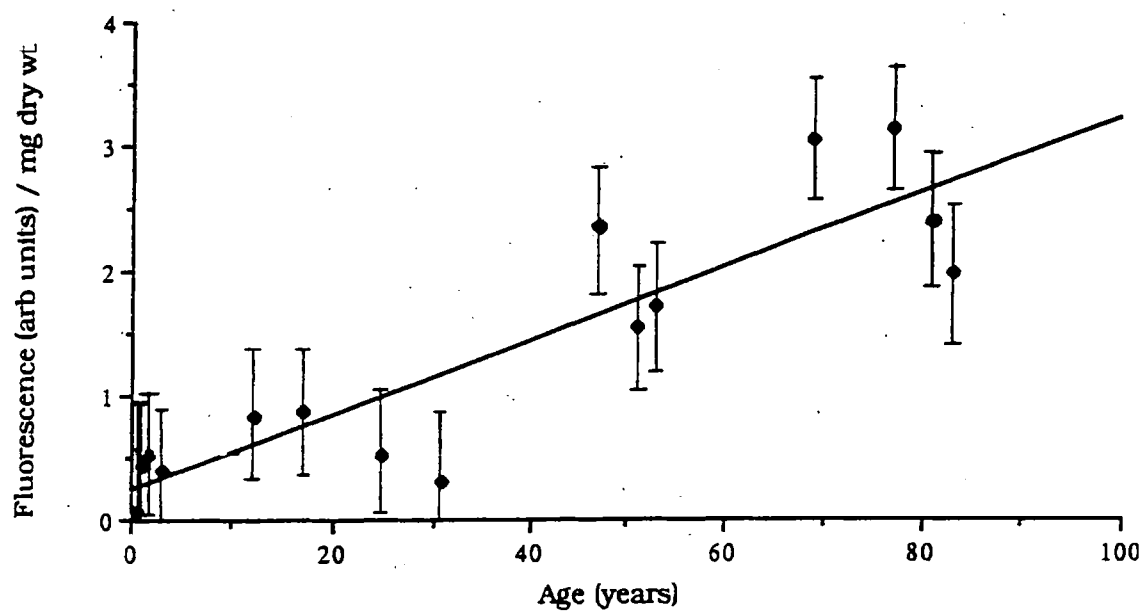


Figure 5.11 Change in fluorescence of scleral collagen with age. A linear increase is observed in the fluorescence of scleral collagen with sample age ($r=0.89$)

However, in human corneas, this concentration of PEG produced hydrations of 2.5 (i.e. below 3.2, the value taken to represent approximately physiological hydration). The results are presented in Figures 5.3 and 5.4. In Figure 5.3 the intermolecular unit cell increases with age; the straight line through the data corresponds to an increase of only 3% from $2.53 \pm 0.10\text{nm}^2$ (birth) to $2.62 \pm 0.19\text{nm}^2$ (age 90). However, in this case there were fewer data points at the lower end of the age range, and from Figure 5.1 the younger corneas tend to show lower intermolecular spacings. In Figure 5.4 the fibril unit cell appears to decrease slightly with tissue age, and in this case a straight line through the data represents a reduction of 8%, from $2791 \pm 35\text{nm}^2$ (birth) to $2555 \pm 20\text{nm}^2$ (age 90).

In a later study (Figures 5.5 and 5.6) the corneal hydrations were set to approximately 3.4 using 1.5% PEG. The straight line through the data of Figure 5.5 corresponds to an increase of 14% in the intermolecular unit cell, this is an increase from $3.04 \pm 0.15\text{nm}^2$ (birth) to $3.46 \pm 0.25\text{nm}^2$ (age 90). Figure 5.6 shows that there is a reduction in the interfibrillar unit cell as did Figure 5.4. However, at a hydration closer to the physiological level, the reduction in the fibril unit cell is greater. A straight line through the data points implies a reduction of 15% from $3550 \pm 20\text{nm}^2$ (birth) to $3018 \pm 8\text{nm}^2$ (age 90). The calculation of correlation between the (interfibrillar spacing)² and the age of the tissue gives a linear correlation coefficient of $r=-0.84$.

Figure 5.7 shows the increase in the intermolecular unit cell of human scleral collagen from birth ($2.65 \pm 0.11\text{nm}^2$) to 90 years of age ($3.19 \pm 0.18\text{nm}^2$). This implies that the intermolecular unit cell increases by 20%, a greater increase than that found in human cornea across the same age range.

In the corneal stroma, the TBA assay shows that there is an initial increase in the glycation of collagen with age (Figure 5.8). After the age of about 50 years, the curve appears to level off suggesting the attainment of an equilibrium or steady-state with respect to extracellular glucose concentration and possibly an equilibrium between the amount of AP and

AGE. Over the age range, the concentration of HMF generated from the Amadori product increases from 6.9 to 10.4 nmol per mg dry weight of tissue. In contrast, scleral collagen shows a progressive increase in glycation across the entire age range, from 0.68 to 4.68 nmol per mg dry weight of tissue (Figure 5.9). The one point on this graph that shows an HMF concentration of approximately 9 nmol/mg dry weight tissue, greater than all the other points, may well represent an individual with impaired glucose tolerance since ageing is known to be associated with impaired control of glucose [Harris et al., 1987]. The difference in the trends in glycation of corneal and scleral collagen may reflect age-related differences in tissue collagen composition or collagen turnover. The corneal stroma is known to have a glucose concentration of $8.8\mu\text{mol/g}$ dry weight tissue [Reim et al., 1967]. With ageing, this may well increase as the body becomes less tolerant to glucose and may contribute to the higher levels of fluorescence found in older individuals.

Figure 5.10 demonstrates the change in the fluorescence of corneal collagen with corneal age. Although a line of best fit has been drawn through the data to indicate the trend in the data points, it is unclear whether the increase is linear and whether it continues beyond the age of about 30 years since the level of scatter in the data increases (the result of individual variation). These data suggest that the level of fluorescent AGE increases in the first three decades of life. The fluorescence of scleral collagen on the other hand, Figure 5.11, does show a clear linear increase across the entire age range from almost zero at birth to approximately 2.6 arbitrary units of fluorescence at 80 years of age. The linear correlation coefficient, r , in this case was 0.89. These data imply that the level of fluorescent AGE increases with scleral age.

Since AGEs have been proposed as cross-linking structures [Sell and Monnier, 1990] and their concentration has been observed to increase in human corneal and scleral collagen, it is possible that, by occurring at specific points between collagen molecules, these structures are responsible for the increase in the dimensions of the molecular unit cell of collagen in

both cornea and sclera (14% and 20% respectively). Any age-related changes in stromal glucose concentration would increase the availability of sugar for glycation. As a result, one would expect the size of the molecular unit cell of collagen to be larger at the older end of the age range which is suggested by Figures 5.5 and 5.7. On re-inspection of these graphs, although there is an overall increase in (intermolecular spacing)², the greatest absolute changes could be occurring between birth and 40 years of age i.e. developmental changes of ageing.

Tanaka et al (1988) concluded from their in vitro studies that, the major structural alteration caused by the glycation of rat tail tendon was in the lateral direction, perpendicular to the fibril axis and represented an increase in the spacing between the collagen molecules in this tissue. They also concluded that only a subset of cross-links causes any significant structural change, i.e. those that lie perpendicular to the molecular axes. It is possible that the increase in spacing between the molecules of collagen in cornea and sclera found in this chapter is the result of glycation-derived cross-links.

The observation of the reduction in the size of the fibril unit cell with corneal age is likely to be linked to alterations in the proteoglycan content or composition of the tissue. Studies to confirm age-related changes in corneal proteoglycan levels were not carried out in this particular project and they are also, as yet, undocumented elsewhere. An electron microscopical study was however performed by Kanai and Kaufman (1973) in which they too concluded that the volume of the fibril unit cell reduces with corneal age. Unfortunately their study included only three corneal specimens and did not produce absolute values for the volume change that occurred. A great advantage of X-ray diffraction is that tissues may be examined in as close to their natural state as possible. Since processing steps such as dehydration and embedding, which are required for electron microscopy, are not needed here, there is a smaller chance of artefactual results.

Chapter 6

X-Ray Diffraction of In Vitro Glycated Tissue Collagen - Results and Discussion

To account for the increased expansion of the molecular unit cell with increasing corneal and scleral age, in vitro studies were performed to see whether the results could be replicated in a 'model system'. On this occasion experiments were performed as a function of time. Since sugar concentrations that were higher than those normally existing within the body were used, the molecular expansions occurred at accelerated rates. Different sugars were employed so that molecular changes resulting from ribation and fructation could also be studied.

6.1 X-ray diffraction and fluorescence studies

In vitro glycation, as described in section 3.3, was carried out with type I collagen (containing small quantities of types III and V) from both human cornea and sclera. Figure 6.1 and Figure 6.2 illustrate respectively the changes in (intermolecular spacing)² and AGE levels (as measured by fluorescence emission) with period of incubation of corneal collagen in the

sugar/buffer mixture. Figures 6.3 to 6.8 show the equivalent changes in sclera at 3 different ages.

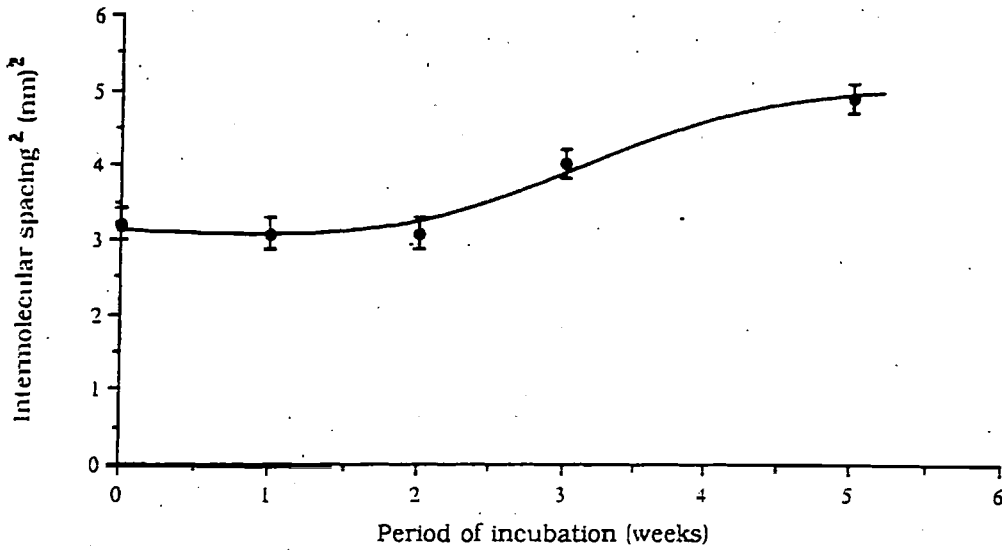


Figure 6.1 Change in (intermolecular spacing)² of in vitro glycated corneal collagen with time (middle-aged corneal specimen used)

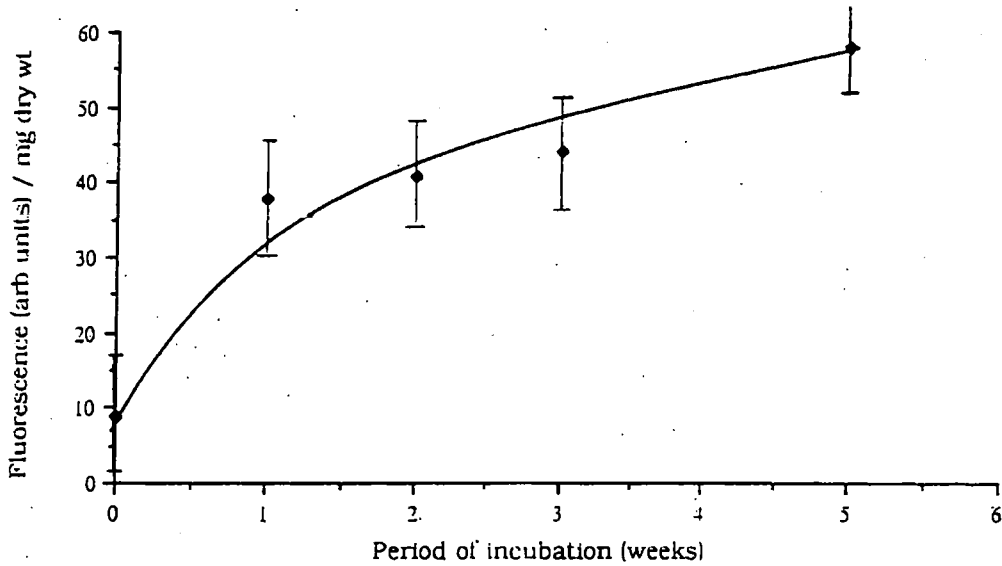


Figure 6.2 Change in fluorescence of in vitro glycated corneal collagen with time (middle-aged corneal specimen used)

The effect of in vitro incubation of scleral type I collagen was investigated using three scleras between the ages of 43-46 years. Three different sugars, namely ribose, fructose and glucose were used and the results are presented in Figures 6.9 and 6.10.

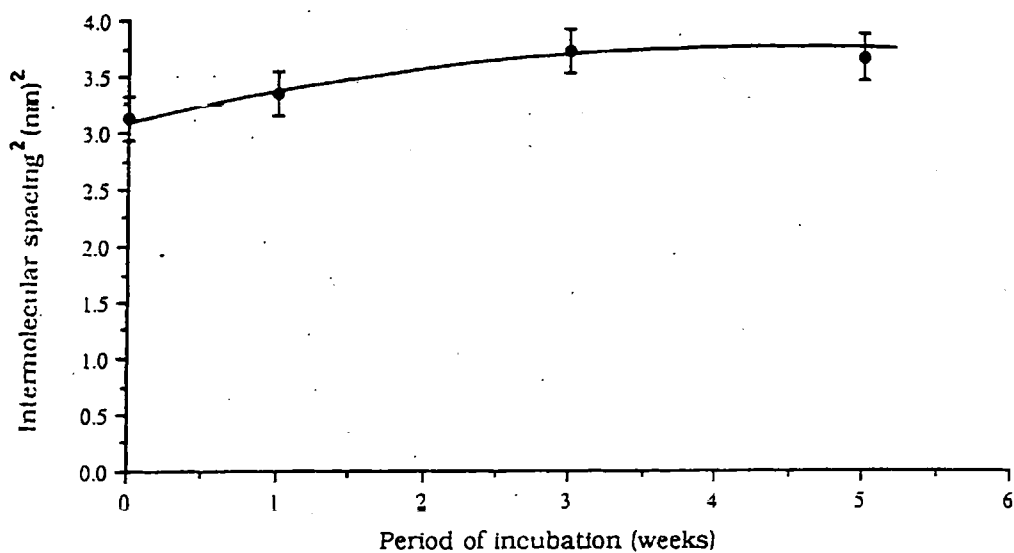


Figure 6.3 Change in (intermolecular spacing)² of in vitro glycated scleral collagen with time (middle-aged scleral specimen)

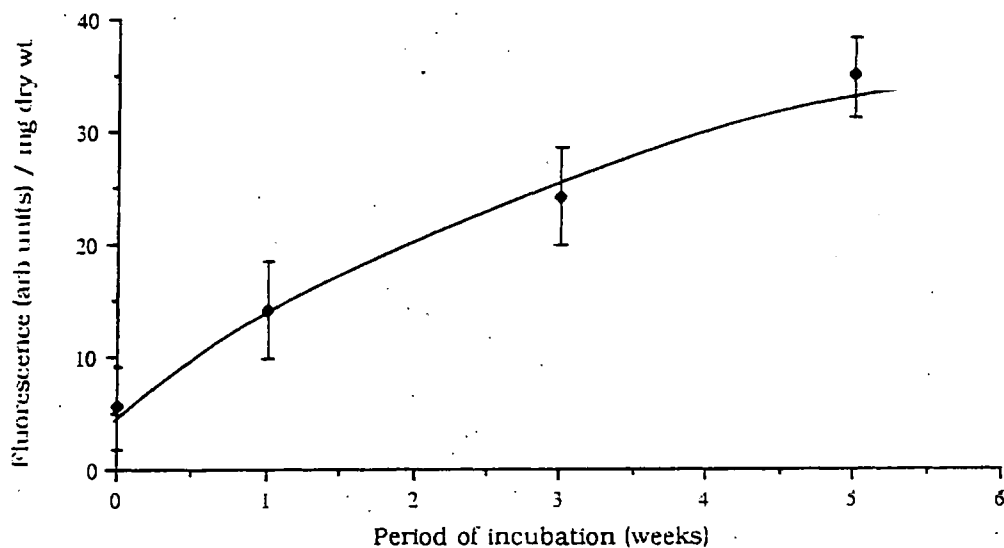


Figure 6.4 Change in fluorescence of in vitro glycated scleral collagen with time (middle-aged scleral specimen used)

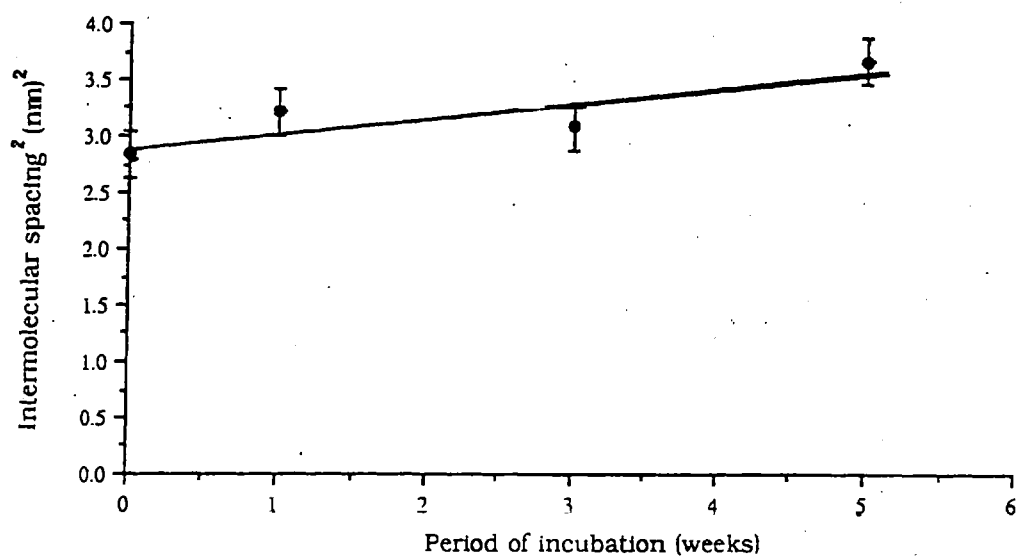


Figure 6.5 Change in (intermolecular spacing)² of in vitro glycated 6wk old scleral collagen with time

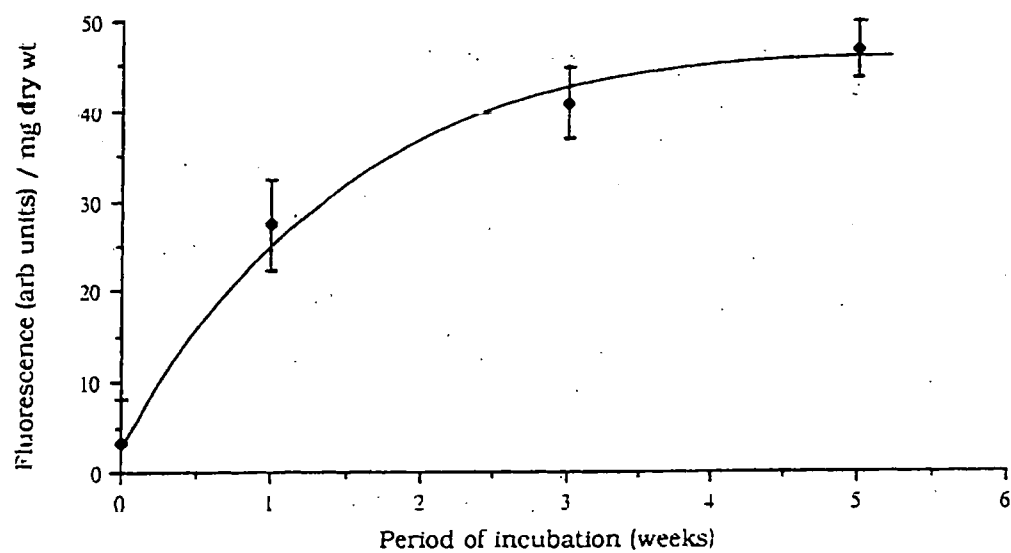


Figure 6.6 Change in fluorescence of in vitro glycated 6wk old scleral collagen with time

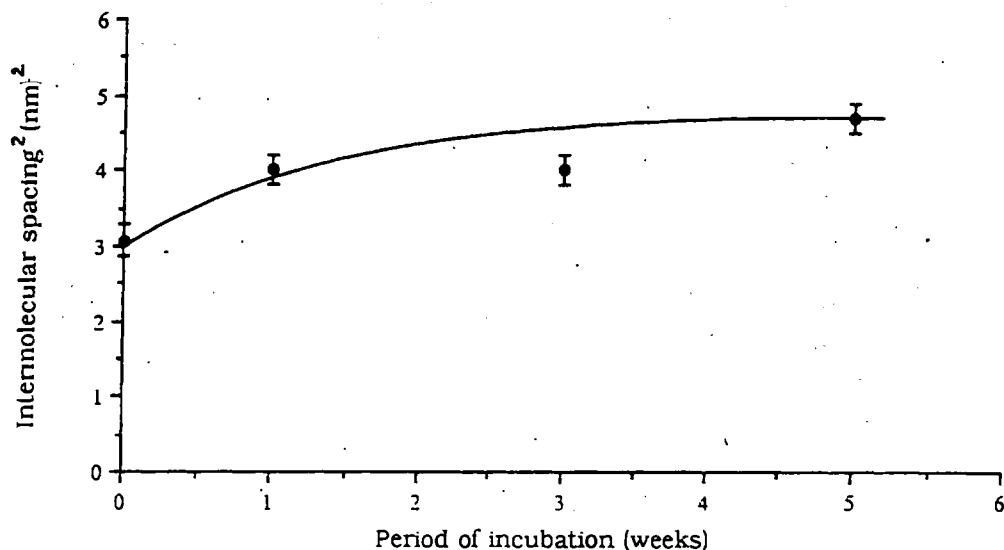


Figure 6.7 Change in (intermolecular spacing)² of in vitro glycated 80yr old scleral collagen with time

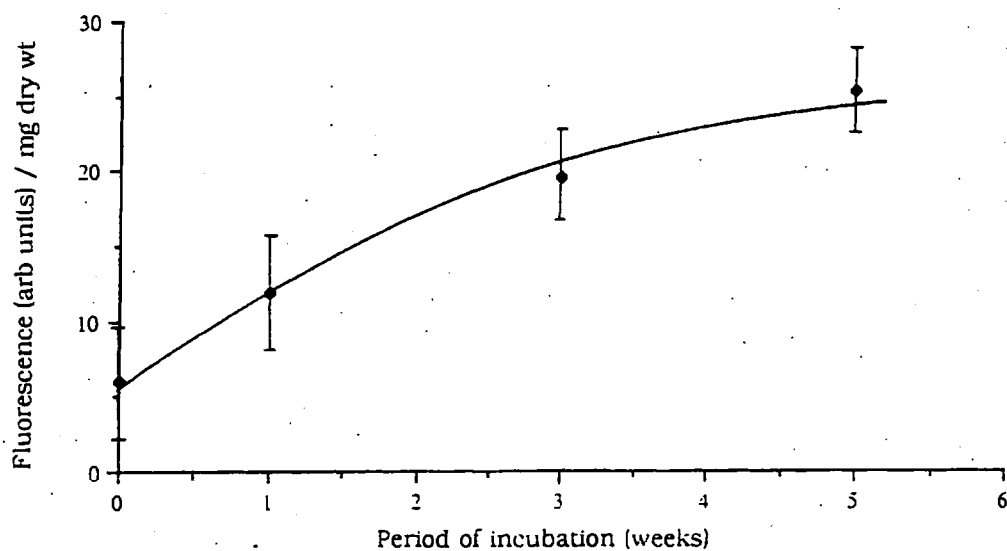


Figure 6.8 Change in fluorescence of in vitro glycated 80yr old scleral collagen with time

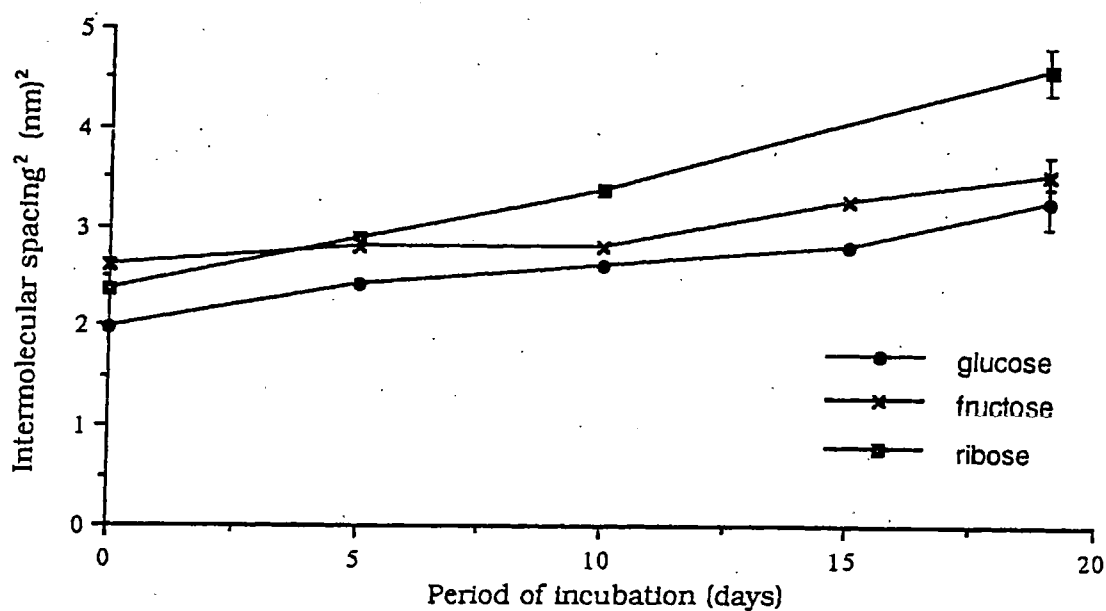


Figure 6.9 Change in (intermolecular spacing)² of in vitro glycated human scleral collagen; collagen taken from 3 middle-aged scleras, sugars used were glucose, fructose and ribose

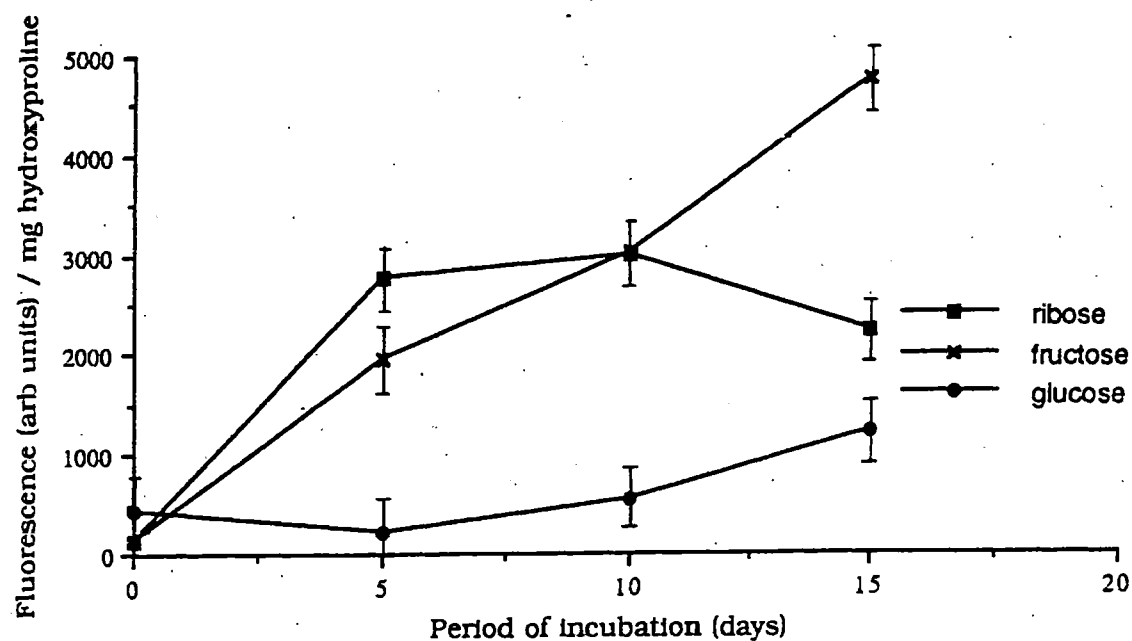


Figure 6.10 Change in fluorescence of in vitro glycated human scleral collagen ; collagen taken from 3 middle-aged scleras, sugars used were glucose, fructose and ribose

6.2 Discussion

Figure 6.1 illustrates that, following an initial lag phase of 2 weeks, the intermolecular unit cell of corneal collagen (taken from a 43 year old specimen) increases from $3.21 \pm 0.25\text{nm}^2$ at time zero (this was the measurement of the control sample which had been incubated at 37°C in the absence of sugar throughout the experiment) to $4.90 \pm 0.51\text{nm}^2$ after a period of 5 weeks incubation with 0.5M glucose. Similarly the corneal fluorescence, reflecting the level of AGE (Figure 6.2), increases from 8.89 (at time zero) to 58 arbitrary units/mg dry weight collagen after 5 weeks exposure of the collagen to glucose. The corresponding sclera of this 43 year-old sample was also used in the in vitro investigation and produced a change (Figure 6.3) in the volume occupied by the scleral collagen molecules of 14% from $3.13 \pm 0.20\text{nm}^2$ (time zero) to $3.66 \pm 0.33\text{nm}^2$ (5 weeks). Figure 6.4 shows that the level of collagen fluorescence (at $370\text{nm}/440\text{nm}$) in this sclera also increased with period of incubation.

Figure 6.5 shows the change in the volume of the intermolecular unit cell of scleral collagen from a 6 week old human sclera. Overall, there is an increase in the dimensions of the molecular unit cell from $2.84 \pm 0.23\text{nm}^2$ (time zero) to $3.66 \pm 0.20\text{nm}^2$ (at 5 weeks incubation) i.e. an increase of 22%. The level of fluorescence, given in Figure 6.6, also increases from zero to 5 weeks producing a very similar curve to that obtained with middle-aged scleral collagen.

Figure 6.7 shows the intermolecular unit cell of collagen from an 80 year old sclera. Again a rapid increase in volume is observed from zero to 1 week however, between 1 and 3 weeks of incubation, the volume of the intermolecular unit cell remains fairly constant. From zero to 5 weeks of incubation the overall increase is from $3.08 \pm 0.26\text{nm}^2$ to $4.70 \pm 0.72\text{nm}^2$ respectively, an expansion of the molecular unit cell of 34%.

Figure 6.8 illustrates the same trend as did Figures 6.4 and 6.6, that the level of AGE increases from zero to 5 weeks of incubation.

From the observed changes in the (intermolecular spacing)² of human

collagen taken from sclera of three different ages, there is no apparent relationship between the age of the collagen and the size of the expansion of the molecular unit cell. This suggests that collagen in younger individuals is no more susceptible to glycation than collagen in older individuals. Comparing the change in the molecular unit cell of a middle-aged cornea and its corresponding sclera, i.e. Figures 6.1 and 6.3, the expansions of 35% and 14% respectively imply that corneal collagen undergoes greater molecular change than does scleral collagen over a specified time period. Although more sets of data are required to confirm whether this is happening, it is interesting to note that at 43 years of age, from the lines of regression in Figures 5.5 and 5.7, the calculated values for (intermolecular spacing)² in cornea and sclera are 3.25 and 2.91nm² respectively. It is difficult to compare the level of AGE found in the 43 year old cornea and sclera (Figs 6.2 and 6.4) since the technique used does not provide us with an absolute concentration of AGE, hence it can only be commented that the level of fluorescent AGE increases in both tissues continuously from zero to 5 weeks incubation. In both cases there is a rapid initial reaction followed by a slower reaction phase. This could be due to a change in collagen conformation eventually exposing the slower reacting sites on the collagen molecules.

In vitro investigations of the glucation, fructation and ribation of human scleral collagen produced the preliminary results illustrated in Figures 6.9 and 6.10. All three sugars produce an increase in the dimensions of the molecular unit cell of scleral collagen between zero and 20 days incubation. The initial rates of increase (between zero and 5 days) in the (intermolecular spacing)² are as follows : 0.09nm²/day (glucose), 0.04nm²/day (fructose), 0.11nm²/day (ribose). These rates suggest that ribose reacts initially at a faster rate than the other sugars. The rates of increase between 5 and 10 days also suggest that ribose is reacting faster than both fructose and glucose and all the rates have slowed down, 0.04nm²/day (glucose), 0nm²/day (fructose) and 0.10nm²/day (ribose). The rates of change between 15 and 20 days were 0.12nm²/day (glucose), 0.07nm²/day

(fructose) and $0.50\text{nm}^2/\text{day}$ (ribose). The corresponding fluorescence of the scleral collagen samples increases with the period of incubation with the three sugars used. Up to 10 days, the graph suggests that ribose and fructose are both reacting at considerably faster rates than glucose.

Following the 10 day incubation period, glucose continues to produce the least fluorescence and fructose produces the greatest. It is difficult to attach any significance to the apparent drop in fluorescence with ribose at 10 days since the accuracy of the fluorescence measurements cannot be quantified, hence it is only the continuing trends that can be commented on.

The expected order of rate of reaction would be ribose>fructose>glucose.

The pentose ring structure of ribose cannot react with amine groups in the triple helical collagen molecule since the carbonyl group is unavailable on this sugar in this form. In solution however, a significant proportion of ribose exists in the straight chain form to allow ribation to occur at a faster rate than fructation and glucation. A greater proportion of fructose than glucose exists in the straight chain form in solution since the ring structure of glucose is very stable. After 10 days fructose appears to be producing higher levels of fluorescence than both ribose and glucose. There is a chance that secondary protein glycation is occurring i.e. fructation of the AP to produce AGEs [Ahmed, 1992]. The changes in intermolecular spacing and fluorescence appear also to run in parallel with the exception of fructose after prolonged incubation. At this point it is possible that fructation is producing fluorophores that do not affect intermolecular spacing. Obviously, more data are required to confirm the rate of reaction observed in this study. However, although these data are preliminary, they are the first to have been obtained with scleral collagen and the first to have been expressed as an effect on the intermolecular unit cell. With reference to the study of the effects of different sugars, comments have been made on the observed trends since it is difficult to comment on individual data points because three different scleras were used and, although they were of similar ages, individual variations undoubtedly existed.

Most in vitro glycation studies by other workers have not incubated

proteins beyond 4-5 weeks. This study extended the incubation of human scleral collagen with fructose to a period of 6 weeks. Three donor scleras were used (aged 6 weeks, 43 years and 80 years) and, interestingly beyond 5 weeks of incubation, the (intermolecular spacing)² of the collagen dropped along with the level of fluorescence (Figure 6.11). Although physiologically this reduction in (intermolecular spacing)² may not be of considerable importance, it was interestingly observed several times.

It is unclear as to why this should occur but it suggests that the in vitro conditions (using higher concentrations of sugar than would otherwise be found in the euglycaemic individual) at 6 weeks result in a breakdown of the AGE cross-linking produced over the first 5 weeks. The result is possibly a more stable structural arrangement.

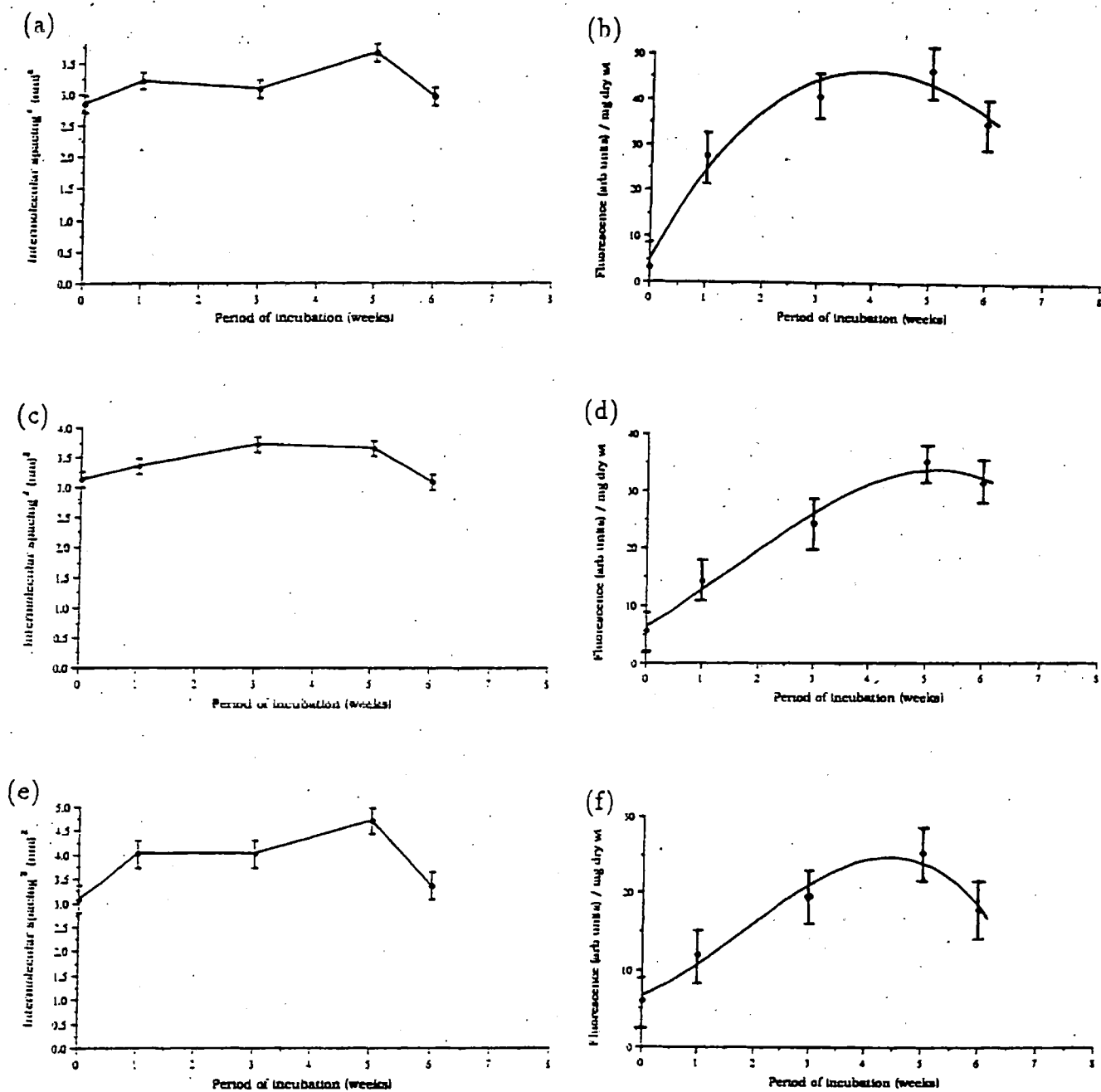


Figure 6.11 Extended in vitro incubation of scleral collagen. (a) and (b), 6 week sclera; (c) and (d), 43 year old sclera; (e) and (f), 80 year old sclera

Chapter 7

Investigation of Different Collagen Sources - Results and Discussion

The previous chapters have been concerned with the alterations in type I collagen from the human cornea and sclera. Type I collagen from various different tissues is supplied by the Sigma Chemical Company. Type I collagen from bovine tendon, calf skin, human placenta and rat tail tendon was investigated along with type IV collagen from human placenta. These collagens were glycated in vitro to compare their susceptibility to sugar attack.

7.1 Glycation and fluorescence

The levels of glycation as measured by the TBA assay are presented as nmols of Amadori product per mg of collagen in Table 7.1 and the corresponding levels of AGEs are illustrated in Table 7.2. The results are also represented graphically by Figures 7.1 - 7.4, curves were fitted according to the method of least squares. Table 7.3 is a summary of the increase in the level of glycation between 0 and 30 days of incubation of the different collagens.

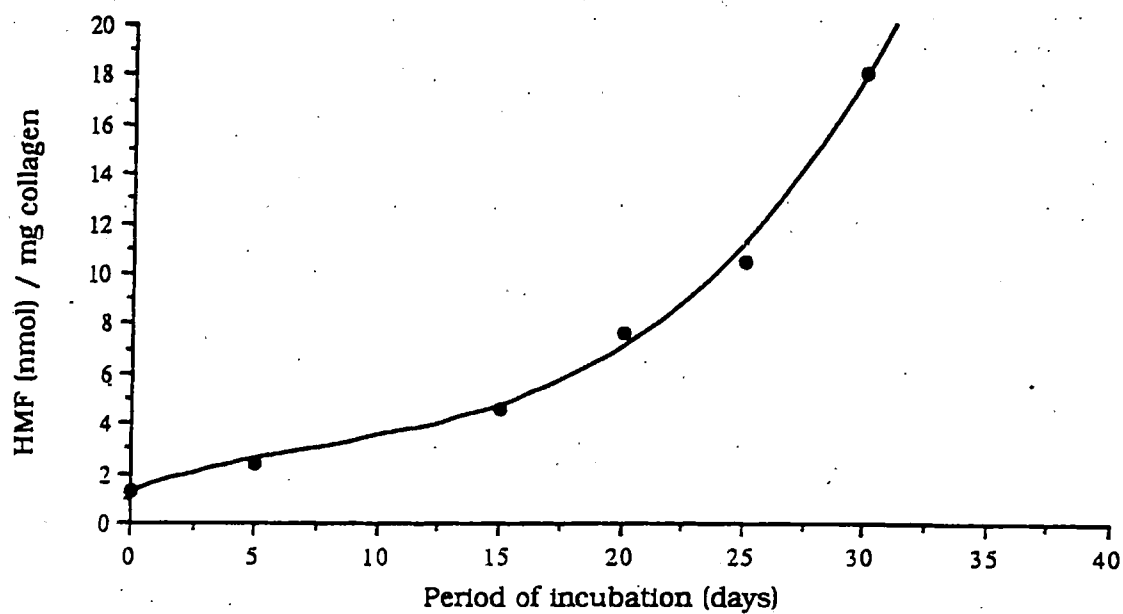


Figure 7.1 Type I collagen from calf skin - Change in Amadori product with time

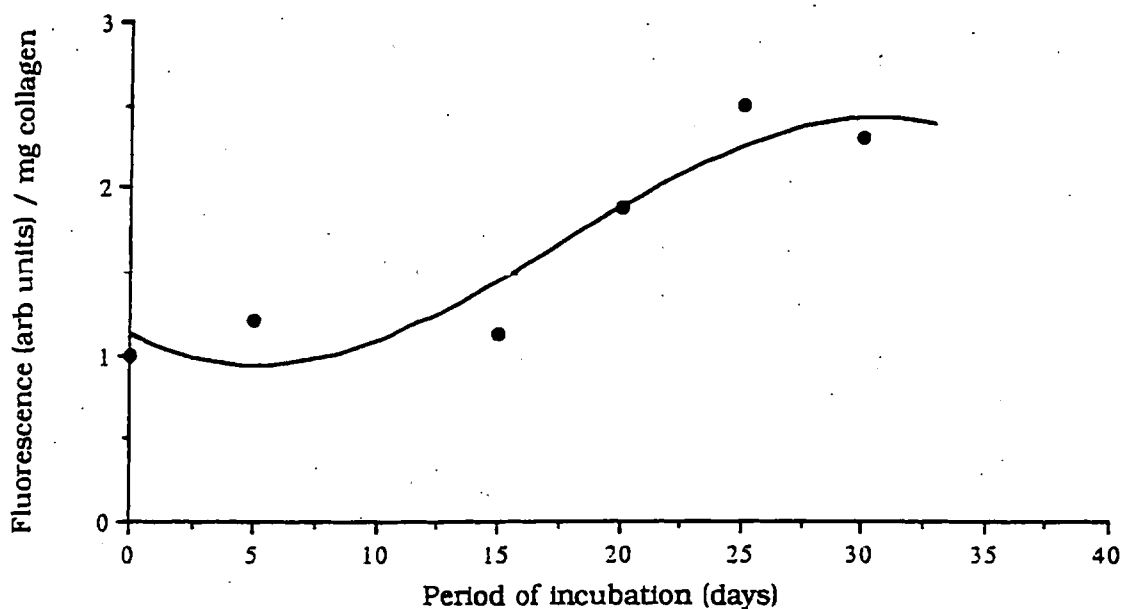


Figure 7.2 Type I collagen from calf skin - Change in fluorescence with time

Time of incubation	I calf skin	I bovine tendon	I rat tail	IV human placenta
0	2.68	1.24	1.92	3.02
5	3.71	2.82	-	-
10	5.02	6.19	-	-
20	9.14	8.11	-	-
30	14.78	12.85	25.70	22.00

Table 7.1 Glycation of collagen types I and IV as measured by the TBA assay ; glycation is expressed as HMF (nmol)/mg collagen, time of incubation is expressed in days

Time of incubation	I calf skin	I bovine tendon	I rat tail	IV human placenta
0	3.38	1.33	0.36	0.44
5	4.80	1.09	-	-
10	5.80	1.23	-	-
20	3.30	2.22	-	-
30	3.73	4.72	2.16	3.22

Table 7.2 The levels of fluorescence resulting from in vitro glycation of collagen types I and IV; fluorescence is expressed as arbitrary units/mg collagen, time of incubation is expressed in days

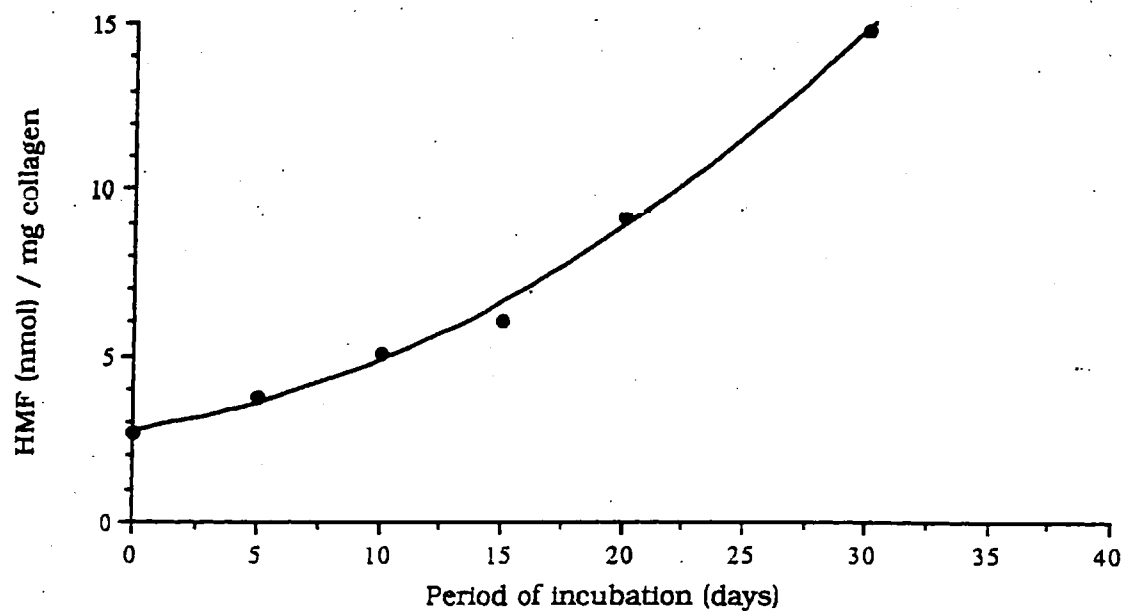


Figure 7.3 Type I collagen from bovine achilles tendon - Change in Amadori product with time

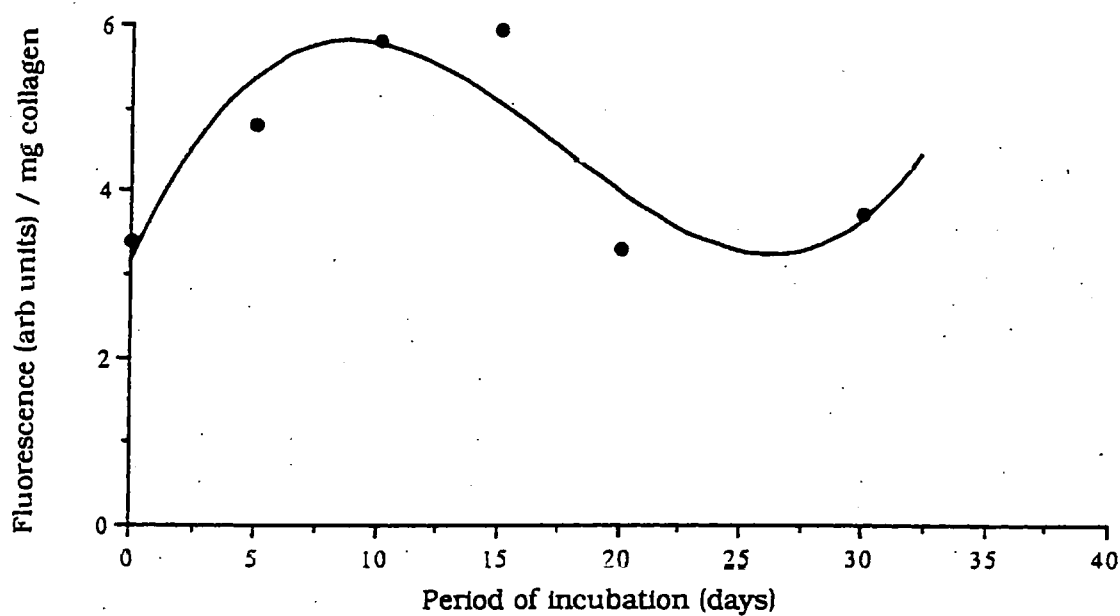


Figure 7.4 Type I collagen from bovine achilles tendon - Change in fluorescence with time

Rat tail tendon is the most likely of all the tissues investigated to contain predominantly type I collagen, hence the Sigma preparation of this type I collagen will be pure type I compared with the other type I sources which are more likely to be collagen mixtures.

From Table 7.1, a comparison of type I collagen from calf skin with that from bovine tendon shows the former to be more highly glycated (in vivo) at time zero than the latter and it also achieves a greater level of glycation at every time point (with the exception of 10 days incubation). Type IV collagen shows a greater level of in vivo glycation (at time zero) and, after 30 days, becomes more glycated than type I from both calf skin and bovine tendon. Type I collagen from rat tail, assumed to be the purest form of type I collagen as supplied by Sigma, shows an even greater level of glycation than type IV after 30 days exposure to sugar. Type IV collagen is a basement membrane collagen and in human placenta would be in close proximity of a rich blood supply hence a good sugar source. This particular collagen type would, in this case, be expected to be more susceptible to glycation than type I collagen. The differences in glycation of these two collagens may reflect differences in purity.

The fluorescence values (Table 7.2) also reflect a greater production of fluorescence at each point of incubation of type I collagen from calf skin with sugar compared with that from bovine tendon. However, after 30 days, the level of fluorescence is highest for bovine tendon type I collagen. After 30 days incubation with 0.5M glucose (Table 7.3) type I (from rat tail) and type IV (from human placenta) produce the largest absolute increases in HMF concentration whilst type I from calf skin and bovine tendon, produces a smaller change.

The in vitro studies (described in Chapter 6), using type I collagen from human cornea and sclera, showed an increase in the level of fluorescent AGE with period of incubation. The Sigma type I collagen, obtained from bovine achilles tendon, calf skin and rat tail demonstrate a similar increase. All the collagens demonstrated an increase in the level of AP with period of incubation suggesting that the level of glycation increased with time in each

<u>Collagen type</u>	<u>Glycation after 30 days</u>
I (rat tail)	23.78
IV (human placenta)	18.98
I (calf skin)	12.10
I (bovine tendon)	11.61

Table 7.3 The increase in the level of glycation after 30 days of exposure to sugar; glycation expressed as HMF (nmol)/mg collagen

case (Table 7.1). Similarly they all showed an increase in the level of fluorescence (with the exception of type I collagen from calf skin which showed a peak fluorescence at 10 days), possibly reflecting increasing levels of AGE, with time.

Chapter 8

Inhibition of Pre- and Post-Amadori Reactions - Results and Discussion

The initial reaction between collagen and a sugar molecule produces a labile Schiff base adduct. An Amadori rearrangement leads to the formation of a relatively stable Amadori product. Through a series of rearrangements, dehydration and hydrolysis reactions, the Amadori adduct is able to yield highly reactive dicarbonyl sugars such as 1- and 3-deoxyglucosone. These dicarbonyl sugars are precursors of fluorescent AGEs. The formation of AGEs (both fluorescent and non-fluorescent) can be stimulated by oxygen and catalysts of oxidation reactions, such as iron and copper ions. It is now thought that the only nonenzymatically formed, carbohydrate-derived products known to accumulate in tissue proteins with age, are products of both glycation and oxidation reactions. The interplay between glycation and oxidation - 'glycooxidation' of a protein - may be of more importance than either of them alone.

The reactions occurring before the formation of the Amadori product are referred to as pre-Amadori reactions and are essentially non-oxidative, those occurring after the rearrangement are known as post-Amadori reactions, these are likely to follow both oxidative and non-oxidative pathways. The inhibition of these reactions has been investigated.

8.1 Results

In this study, corneal tissue from three corneas of similar ages (46-53 years), was incubated with 0.5M fructose in phosphate buffer at 37°C and a specified inhibitor (the concentration of the inhibitor in each case was 25mM). The experiment was designed to look for changes in the intermolecular unit cell with the time of incubation in fructose with and without inhibition. The results are presented in Table 8.1.

Reading down each column, the table shows that all three compounds, aminoguanidine (AG), acetylsalicylic acid (ASP) and diethylenetriaminepentaacetic acid (DETAPAC), successfully inhibited the expansion of the collagen molecular network associated with fructation. In the absence of an inhibitor, the unit cell increased by 30% after 24 days, compared with 0% (AG), 11% (ASP) and -11% (DETAPAC). At time zero, the unit cell in the control was larger than those in the other three corneas. This was probably because the closest control sample available at the time was 10 years older than the test samples.

8.2 Discussion

The apparent 17% reduction in the molecular unit cell during the early stages of incubation with AG is significant ($p < 0.05$). DETAPAC also produces a significant reduction ($p < 0.05$) in the unit cell size of 10% over the same period whilst ASP produces no significant change. Fructose was unable to cause an expansion of the corneal collagen molecular network in the presence of the three inhibiting compounds used. This suggests a hindrance in the production of new cross-links. Aspirin (acetylsalicylic acid) (Figure 8.1) acts by blocking the free amino groups of corneal collagen by acetylating them. This subsequently prevents the production of AP and AGEs i.e. an inhibition of both glycation and AGE formation. Aspirin has been shown to reduce the glycation of albumin and haemoglobin [Rendell et al., 1986] and also lens protein [Swamy and Abraham, 1988].

Aminoguanidine, on the other hand, has been suggested to block free carbonyl groups [Brownlee et al., 1988]. Free carbonyl groups exist within the sugar used (i.e. fructose) and within reactive dicarbonyl compounds, such as 3-deoxyglucosone, produced from the AP. The result is an inhibition of post-Amadori reactions whereby, glycation may be affected if the aminoguanidine attacks the sugar, and cross-link formation is prevented. This is illustrated in Figure 8.2.

DETAPAC (Figure 8.3) is a known metal ion chelator [Hunt et al., 1988].

Inhibitor	AG	ASP	DETAPAC	no inhibitor
Days of incubation				
0	2.69	2.69	2.69	3.08
3	2.22	2.69	2.66	-
6	2.22	2.69	2.43	3.35
15	2.22	2.69	2.69	4.01
24	2.69	2.99	2.40	4.01

Table 8.1 The effect of various inhibitors on the intermolecular volume of corneal collagen; intermolecular volume expressed as (Intermolecular Bragg spacing)² nm²

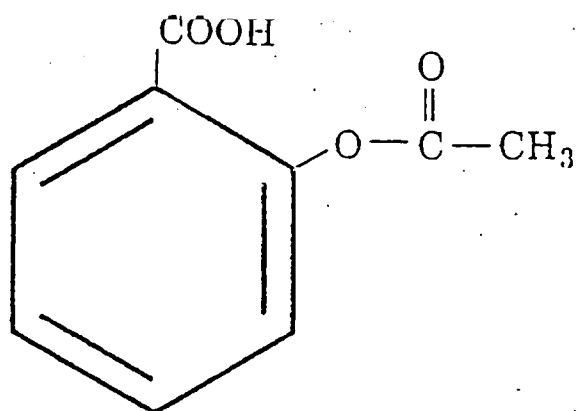


Figure 8.1 The chemical structure of acetylsalicylic acid (aspirin)

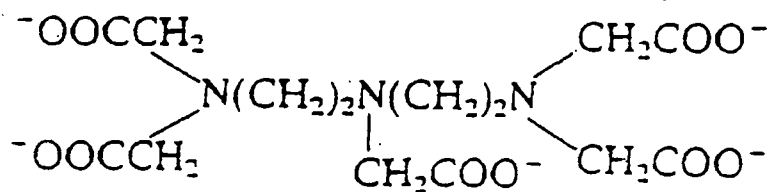


Figure 8.3 The chemical structure of diethylenetriaminepentaacetic acid (DETAPAC)

Since one route to AGE formation is suggested to involve autoxidation reactions catalysed by metal ions, DETAPAC would inhibit these reactions by removing the catalysts. From the study in this chapter, DETAPAC effectively prevented the increase in molecular unit cell dimensions that otherwise occurs with sugar.

It must be emphasised that this particular study has been a preliminary one and is the first to express the effects of inhibition of glycation and AGE formation on intermolecular volume. Although the effect of the inhibitors alone has yet to be studied, the results presented at this stage strongly suggest that all three compounds successfully prevented the increase in the molecular unit cell that occurs with fructose alone.

Chapter 9

Final Discussion

The work of this thesis, as outlined in the aims and objectives, has revealed several age-associated changes in corneal and scleral collagen. The structural alterations that have been observed within the collagen have been reproduced by in vitro glycation experiments as hoped. The concentration of AGEs has also been shown to increase in corneal and scleral collagen with age and could be linked to cumulative glycation-based changes and changes in glucose levels on ageing of an individual. Although these changes are small, they have now been consistently observed and are found to be greatly accelerated under diabetic conditions [Brennan, 1989]. These structural alterations have been discovered and quantified as part of this thesis using a high intensity synchrotron source. Since this technique, unlike many others, allows tissue to be kept in as natural state as possible, the observations are more accurate than, for example, electron microscopy used by other workers.

The X-ray diffraction studies have determined the (intermolecular spacing)² to increase with age in corneal and scleral type I collagen respectively. In conjunction with this, the level of glycation also increases in both tissues. In the cornea, the amount of collagen glycation appears to reach a plateau after approximately 40 years of age and, on reinspection of Figure 5.5, the size of the molecular unit cell may also do the same. The level of fluorescence, at 370nm/440nm, also appears to increase with tissue age

although the increase in scleral collagen is more noticeable ($r=0.89$). From the studies performed, the production of AGE reflects production of 'new' cross-links. It is very likely, from the observations, that such structures are responsible for the progressive alteration in the collagen molecular unit cell of both tissues and that the observed expansion represents the average spacing of normal and cross-linked molecules.

The cross-linking structure has been quantified through fluorescence using excitation/emission wavelengths characteristic of a known fluorescent cross-link identified in human skin collagen. Pentosidine is the only well-characterised cross-link formed in the Maillard reaction of sugars with proteins under physiological conditions. The wavelengths chosen for the study were not optimum for pentosidine (since it is likely that non-pentosidine cross-links are involved) but appeared to be the most sensitive to glycation-induced change. The pentose ring structure is coupled to both an arginine and lysine residue. The involvement of a pentose in the cross-link would be unexpected since, for one reason, the total plasma concentration of pentoses in humans is 100 times less than that of glucose. This observation may, however, have evolutionary significance since rodents have very high ribose concentrations and a short lifespan [Heaf et al., 1976] - this could be linked to accelerated ribose-mediated cross-linking. Ribose may be the major precursor of pentosidine in vivo. It is true that, in a diseased kidney, this sugar would be cleared at a slower rate. Any accumulation of ribose, or any other sugar for that matter, would potentially allow protein glycation followed by AGE production. Since pentoses in aqueous solutions contain a higher percentage of the non-cyclic carbonyl (the reactive group of the sugar molecule involved in glycation) than hexoses, they are more susceptible to autoxidation [Bunn and Higgins, 1981] - this may also lead to AGE formation. The concentration of pentosidine in human lens and skin collagen has been found to increase gradually with age. However, the concentration observed in older tissues has not been reported to reach significantly high concentrations. It has been predicted that, the concentration of pentosidine in human skin collagen of

approximately 90 years age, could reach a level at which it could increase the cross-linking of triple helical collagen by 10% [Sell and Monnier, 1990]. The results of this thesis suggest alterations in the molecular unit cell size in the order of 14% (corneal collagen) and 20% (scleral collagen). Although concentrations of pentosidine may be low, it is highly likely that other sugar-derived cross-links are present at the same time in vivo.

Recently it has been reported that the extent of glycation of lens proteins does not increase with age [Patrick et al., 1990]. The discrepancies may lie in differences in assay procedures and sample populations used by different laboratories. The TBA assay used in this thesis is known to be subject to interference by 6-carbon sugars [Schleicher and Wieland, 1981] with acid-labile glycosidic bonds. The major source of these sugars are the corneal proteoglycans, however, prolonged extraction in 4M guanidine hydrochloride should overcome this problem by removing the proteoglycans [Wall, 1990]. Incomplete removal of proteoglycans or other contaminating substances results in an interference with the TBA assay of less than 10% since borohydride reduction experiments have shown that, more than 90% of the colour generated in the TBA assay is derived from non-enzymically glycosylated collagen [Ahmed et al., 1992]. Under our conditions therefore, the TBA assay results can be taken to indicate an age-related increase in AP (i.e. glycation). The autoclave version of the TBA method by Parker et al was used in this thesis. It has the advantage of requiring only 1 hour of boiling whereas earlier versions involved between 4 and 6 hours, hence they were time-consuming. The Parker method was modified further during this thesis to increase the assay sensitivity and the yield of HMF (an indirect measure of AP). During this thesis, the method was also adapted for use with a microplate autoreader, resulting in a simple and rapid microassay. The experiments concerned with the effect of different sugars on the molecular unit cell of human scleral collagen (Chapter 6) suggested that ribitation and fructation induced greater alterations in unit cell dimension than did glucation. In vitro studies using radiolabelled sugars have shown that fructose indeed reacts faster than glucose and forms the SB

approximately eight times faster than glucose with haemoglobin [Bunn and Higgins, 1981]. Fructose has also been shown to induce AGE formation up to ten times faster than glucose in vitro with albumin [Suarez et al., 1989]. There are regions in the body where fructose can accumulate and in these regions the sorbitol pathway exists. This pathway has been identified in corneal epithelium and is responsible for converting excess glucose to sorbitol and fructose as illustrated in Figure 9.1.

Since impaired glucose tolerance occurs with age [Harris et al., 1987], there is a chance that the sorbitol pathway may become more active. The production of fructose would increase, which in turn would increase the likelihood of fructation of a long-lived protein such as collagen. Direct evidence of fructation has been reported by McPherson et al (1988) who suggested that 20% of the AP in lens protein is derived from fructose. Sorbitol would also induce cellular damage since it would accumulate intracellularly to produce severe osmotic alterations - this may play a role in corneal epithelial diabetic abnormalities.

The major structural alteration in collagen caused by glycation is suggested by Tanaka et al (1988) to be in a lateral direction, perpendicular to the fibril axis. These workers suggested an expansion of the lateral dimensions (Figure 9.2) of a unit cell describing the three-dimensional crystalline packing of rat tail tendon collagen. The increase in spacing between the collagen molecules (of approximately 12%) was confined to a single plane of the structure - essentially the plane at right angles to the fibril axis. The plane was one of the three major planes of the proposed quasi-hexagonal structure of rat tail tendon collagen.

The expansion of the molecular unit cell of both corneal and scleral collagen is not simply an effect of changes in tissue hydration resulting from the presence of free sugar, since the in vitro studies used tissue samples at time zero which were incubated in the sugar/buffer mixture throughout the entire period of the investigation. Alterations in the (intermolecular spacing)² occurred in the tissue samples at subsequent time points. Since the cross-link pentosidine contains a lysine and arginine residue, it is

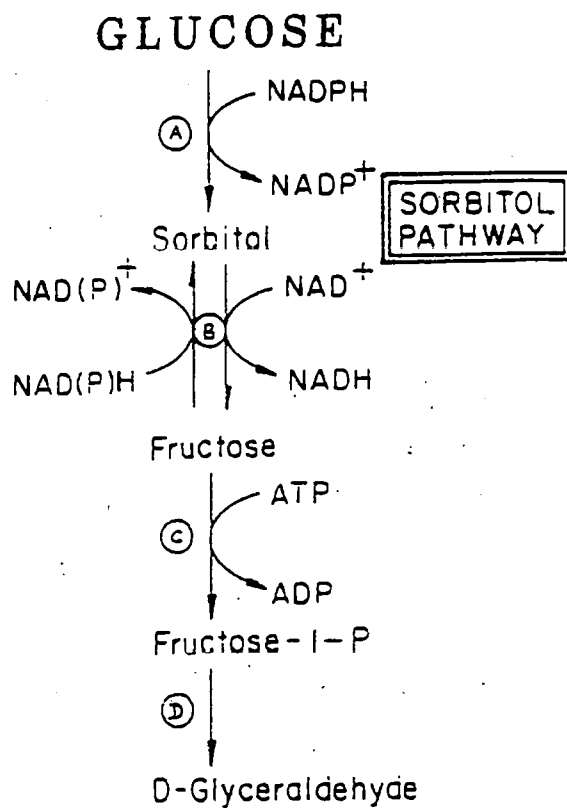


Figure 9.1 A diagrammatic representation of the sorbitol pathway found in corneal epithelium. Enzymes - A, aldose reductase; B, polyol dehydrogenase; C, ketohexokinase; D, aldolase

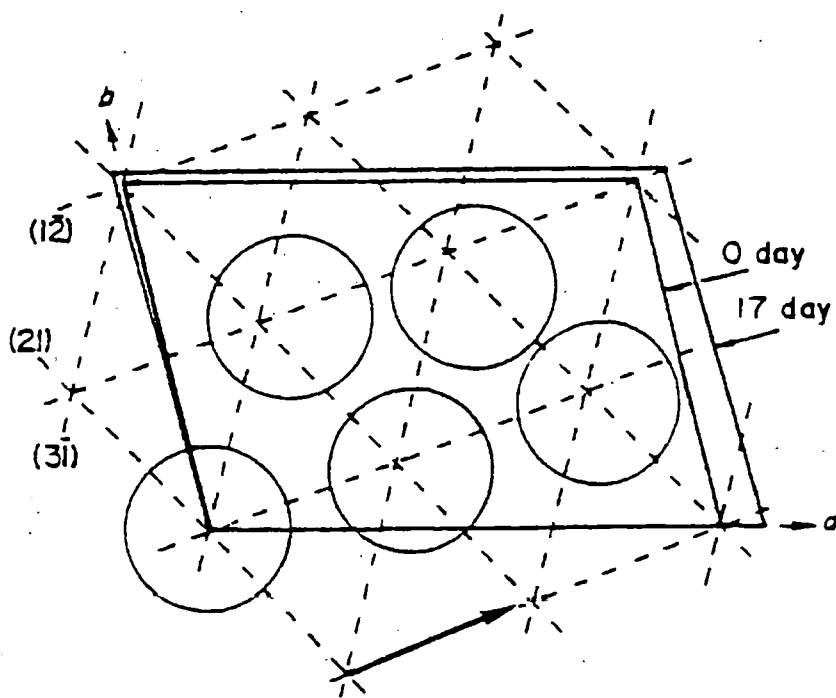


Figure 9.2 Changes in the ab plane of the quasihexagonal unit cell of rat tail tendon collagen after incubation with 0.2M ribose. Unit cells at 0 and 17 days are illustrated, the 'new' positions of the collagen molecules in the native structure are indicated by the circles (reproduced from Tanaka et al., 1988)

likely that it is occurring between adjacent collagen molecules, possibly in the overlap zone of the fibril. This may account for the lateral expansion observed by Tanaka et al. Although this group of workers did not investigate AGEs, they have postulated that molecules could be pushed along a considerable length in directions away from a cross-link site as opposed to the localisation of a cross-link effect. They suggest too that a consequence of this expansion is the entry of water into the fibril. A greater volume of water within the fibril would suggest less water between the fibrils and the overall result would imply a reduction in the interfibrillar distance. This is one of the observations made in this thesis. Sugar-derived cross-links are therefore more likely to occur where lysine, hydroxylysine and possibly arginine residues exist on adjacent molecules at the same axial position and are more or less directly opposite each other. Only those sugar-derived cross-links occurring at right angles to the fibril axis would produce such structural alteration - this implies that only a subset of AGEs is responsible for the changes in the molecular unit cell of collagen, namely, relatively rigid sugar-derived cross-links pushing apart adjacent molecules in the closely packed overlap regions of fibrils. Naresh and Brodsky (1992) have recently suggested that there are differences in the lateral packing of collagen molecules in young and old rat tail tendon. Using X ray diffraction, they found the intensities of the 14-18th order meridional reflections to be sensitive to the conformation of the terminal non-helical peptides (known as telopeptides). The increase in spacing between the collagen molecules may very well result in conformational changes of the relatively flexible telopeptides.

During the investigation of inhibition of glycation and AGE formation, the metal ion chelator DETAPAC was used. Transition metal ions are potent catalysts of autoxidative reactions which can result in AGE formation. The copper concentrations within human cornea and sclera are 10 and 8 $\mu\text{g g}^{-1}$ fresh tissue respectively [de Azevedo and de Jorge, 1965]. The concentrations of such ions may change with age, possibly increase with age since free metal ions are constantly being released during cell death,

phagocytosis and inflammation - this would encourage AGE formation.

Oxygen radical scavengers also exist within the body to limit the biomolecular damage caused by autoxidative reactions. One such scavenger is vitamin C (ascorbate) however, its concentration has been found to decrease in corneas of 40 years upwards [Seidler-Dymitrowska, 1953].

Although natural defence mechanisms limit the accumulation of products of autoxidation, in long-lived proteins, such as collagen, products of oxygen radical reactions can accumulate with time. These products may contribute to the molecular alteration of collagen. Although protein aggregation and the formation of fluorescent cross-links can be induced by free radical attack on any protein, glycated proteins are far more susceptible [Jones and Lunec, 1987].

It was noted, during the *in vitro* investigations in this thesis, that the longer the tissue had been incubated in sugar/buffer mixture, the more brown it appeared in colour. At the same time the tissue became stiffer and more resistant to enzymic digestion. Older corneal and scleral tissues were more difficult to digest with enzymes suggesting extensive cross-linking in older collagen.

Glycation may take place at biologically important sites of the collagen molecule, for example the collagenase binding site or a glycoprotein interacting site. In the glomerular basement membrane of the kidney, the glycation of the main collagenous domain NC1, located at the carboxyl end of type IV collagen molecules, may interfere with the normal assembly of type IV collagen molecules in diabetes and is possibly related to the abnormal basement membrane function in this condition. Cell culture studies by Danne et al (1993) using glomerular epithelial and endothelial cells have shown that these cells, when exposed to 30mM glucose for 60 hours, synthesize type IV collagen at a rate 2-3 times greater than at physiological glucose levels. They also found that the stimulatory effect on type IV collagen production was only slowly reversed after restoration of the glucose to a normal level. It has also been reported that stimulation of collagen synthesis by a high glucose concentration is a self-limited

phenomenon with long-term elevation of glucose even resulting in decreased collagen production [Silbiger et al., 1993]. This implies that turnover slows down and, since collagen is a long-lived protein and AGE formation is a slow process, the likelihood of a cumulative build-up of AGEs is very possible. The production of the SB and the AP from glycation results in changes in the isoelectric point of the collagen, this would in turn alter the molecular conformation. Other workers [Yue et al., 1983] have found that the tensile strength of rat tail tendon collagen alters with glycation. If the fibrils in a bundle become cross-linked by sugar-derived cross-links formed between molecules of neighbouring fibrils, then this cross-linking of adjacent fibrils would result in a larger stress-bearing unit, hence the requirement of a larger force to break the tendon [Bai et al., 1992]. If this were to occur in human cornea and sclera, the rigidity of both tissues would be altered. Glycation appears to be an important factor in mediating alterations in tissue morphology and in altering the biological and mechanical properties of a collagenous tissue, such as cornea or sclera, in ageing.

Although this thesis has not studied the effects of glycation on corneal transparency, the tissue has been observed to develop a brownish colouration. The build up of coloured AGEs would be expected to have some effect on the optical properties of the tissue.

The investigation of the different collagens found that they had different susceptibilities to sugar attack. Type I, from rat tail, and type IV, from human placenta, were more glycated than type I from either calf skin or bovine tendon over a 30 day incubation period. Differences in glycation levels would be expected since the tissues are located in areas where the blood supply varies as does the activity of metabolic pathways.

It has been suggested that the hydroxylysine residues are targets for glycation. Table 9.1 shows several collagen types and the lysine/hydroxylysine/ arginine content of each type. One would expect type IV collagen from human placenta to be more susceptible to glycation in vivo because of its location (in close proximity of a good blood supply) with respect to the other collagens. Table 9.1 indicates that type IV collagen

potentially has a greater number of lysine and hydroxylysine residues for glycation than type I collagen. However, the experimental results in Chapter 7 suggest that type I collagen from rat tail tendon achieves a greater level of glycation than type IV collagen from human placenta after 30 days exposure to sugar. The differences may be a result of the existence of positive groups (on amino acid residues) adjacent to glycation sites which could promote catalysis of the Amadori rearrangement.

Residues/1000 residues	I	III	IV	V
Lysine	25	29	9	18
Hydroxylysine	11	6	49	34
Arginine	50	48	26	47
Total	86	83	84	97
Lysine + Hydroxylysine	36	35	58	50

Table 9.1 The amount of lysine, hydroxylsine and arginine found in collagen types I, III, IV and V (compiled from Burgeson (1982) and Jander et al (1984))

It is likely that the conformation of the molecules (hence the accessibility of

sites for glycation) at the time of glycation, together with the proportions of triple helix and globular domains, play a role in determining why one collagen type should be more reactive than another.

The inhibition of AGE formation was investigated in order to prevent molecular alterations of both corneal and scleral collagen. There is evidence, however, that the body itself is able to trace, capture and degrade proteins containing AGEs. Macrophages are thought to bind and degrade AGE-containing proteins via a specific cell-surface binding protein. The binding protein is able to recognise carbonyl groups and has a molecular weight of 90 kDa [Radoff et al., 1988]. These macrophages have been investigated with respect to proteins such as albumin however, there is no evidence to suggest that they are involved in the same way in cross-linked collagen, indeed it is not known whether they are able to penetrate the corneal tissue.

Glycation has a profound effect on collagen in vitro and, from the X-ray diffraction studies in this thesis, results in age-related alterations in the size of the corneal and scleral molecular unit cell of long-lived collagen. It is likely that the process is one factor in a series of modifications occurring during the process of ageing.

Another structural modification found to occur on the ageing of an individual was the decrease in the fibril unit cell of collagen in the cornea. The 15% reduction from birth to approximately 90 years of age was suggested to be a result of age-related changes in proteoglycan concentration. Although a comprehensive study of proteoglycan alteration with age has not been reported, a decrease in the ratio of proteoglycan to collagen was observed by Scott et al (1981). From the studies in this thesis, it is interesting to note that, even when corneas at each end of the age range are equilibrated to the same hydration, there is a 14% increase in the molecular unit cell and a 15% decrease in the fibril unit cell with age. Both would result in a significant overall decrease in the extrafibrillar space where most of the proteoglycans are situated. The regular interfibrillar spacing of constant diameter fibrils appears to be vital for transparency and

the regular arrangement of the proteoglycans along the collagen fibril must contribute to this. It is suggested that proteoglycan:collagen interactions keep fibrils apart and hold them in definite orientations. Keratan sulphate concentrations have been found to increase with corneal thickness whilst dermatan sulphate decreases. However, a significant thinning of the corneal periphery has been reported together with a decrease in the ratio of peripheral to central corneal thickness after 50 years of age [Martola and Baum, 1968]. Natural age-associated alterations in proteoglycan concentrations may well be responsible for the reduction in (interfibrillar spacing)² with corneal age. From the SDS-PAGE and electron microscope studies on the corneal incubation medium (Chapter 4) indicating that the culture medium did not induce significant proteoglycan loss, the decrease in the size of the fibril unit cell of human corneal collagen is an age-related occurrence.

The effect of glycation on proteoglycan structure was not studied here. However, if there is an alteration in the degree of sulphation, hence charge of proteoglycans, the proteoglycan-collagen interactions would be affected and these would in turn alter the assembly and function of the collagen matrix.

Bai et al (1992) have recently suggested, through electron microscopical observations, that the glycation of rat tail tendon collagen results in close packing of fibrils. Although proteoglycans are commonly associated with maintaining precise spatial relationships between collagen fibrils, it is possible that the process of glycation causes 'fibril fusion' suggesting that cross-linking is occurring between collagen molecules in different fibrils.

This has not yet been observed in other collagenous tissues.

Glycation and proteoglycan composition/type alterations result in changes in electrical charge on the collagen molecule and along the fibril. The outcome would be swelling of the collagen fibrils which would in turn reduce the extrafibrillar space, i.e. a reduction in the size of the fibril unit cell. It is probable that no one phenomenon is entirely responsible for the change in this particular parameter and therefore likely that they each play

a role in this age-related alteration.

9.1 Suggestions for further study

After a study of this type, where most of the parameters investigated are the first to be reported for corneal and scleral type I collagen and for several other collagen types, there is great scope for further work. Amongst the possibilities are the following :

- 1) It would be appropriate to locate the sites of AGE cross-linking along the collagen fibril possibly by investigating changes in the electron density along the fibrils resulting from AGE formation. As yet, no observations have been made concerning changes in the meridional X-ray pattern as a function of age or glycation. Such changes would be expected if the axial electron density of corneal and scleral collagen was affected by cross-linking.
- 2) Since inhibitors of glycation and AGE production have been used to limit the alteration in the molecular unit cell size, it would be of interest to investigate the use of naturally-occurring compounds, such as vitamin C (ascorbate), the concentration of which is known to decrease in human cornea, in the same context.
- 3) An age-related study of changes in the iron and copper ion concentrations of corneal and scleral tissues is required in order to see whether metal-catalysed oxidative reactions are playing a significant role in AGE formation.
- 4) A comprehensive study of the level of proteoglycans in both the human cornea and sclera is required as a function of age, since these macromolecules are known to be involved in interfibrillar separations.
- 5) The effect of glycation on proteoglycan structure and proteoglycan levels needs to be investigated since, any alteration in the charge of glycosaminoglycans, would affect the behaviour of the collagen-containing matrix in both cornea and sclera and other connective tissues.

9.2 Publications

A comparison of proteoglycan arrangement in normal and keratoconus human corneas

Nigel J Fullwood, Keith M Meek, Nageena S Malik and Stephen J Tuft.

(1990) *Biochem. Soc. Trans.* 18, 961-962.

The interfibrillar and intermolecular spacing in corneal collagen as a function of hydration and tissue age

Nageena S Malik, Nigel J Fullwood, Keith M Meek and Rita S Wall.

(1990) *Proc. Int. Soc. Eye Res.* volume VI, 669.

Ageing of human corneal and scleral collagen

Nageena S Malik, Keith M Meek, Gerald F Elliott and Anna J Furth.

(1992) *Invest. Ophthalm. Vis. Sci.* 33, 895.

Synchrotron X-ray diffraction studies of keratoconus corneal stroma

Nigel J Fullwood, Stephen J Tuft, Nageena S Malik, Keith M Meek, Alan E A Ridgway and Rosalind J Harrison.

(1992) *Invest. Ophthalm. Vis. Sci.* 33, 1166-1173.

Ageing of the human corneal stroma: structural and biochemical changes

Nageena S Malik, Stasia J Moss, Nessar Ahmed, Anna J Furth, Rita S Wall and Keith M Meek.

(1992) *Biochim. Biophys. Acta* 1138, 222-228.

Structural and biochemical studies of organ-cultured human corneas

Nageena S Malik, Keith M Meek and Stasia J Moss.

(1993) *Doc. Ophthalm.* In press

A colorimetric microassay for glycated collagen based on the TBA method

Nessar Ahmed, Nageena S Malik and Anna J Furth.

(1992) *Clin. Chim. Acta* 212, 133-139.

Aspirin and other compounds prevent sugar-induced structural alterations
in corneal collagen

Nageena S Malik and Keith M Meek.

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Chapter 10

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